OXIDATIVE FLAVOUR CHEMISTRY AND BIOCHEMISTRY IN PARSLEY.

By

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To Angela, Ashley and Hayley.

Contents

Abstract

Deterioration of flavour quality during processing and storage is often brought about by oxidative processes. These typically involve oxygen or an active form of oxygen in effecting transformation of a wide range of volatile and non-volatile compounds, including key quality chemicals, flavour precursors and antioxidants. To investigate the nature of the chemical and biochemical change within vegetables and herbs, unblanched frozen parsley was selected as a suitable tissue.

The chemical status of parsley during technological processing was determined using novel analytical protocols (SNCVA/SNCNVA) implemented as part of a unified strategy for the quantitative analysis of volatile and non-volatile species. The analysis utilized a single stabilized solution produced from plant tissue, under a regime which minimized isolation stress and artifact formation.

On frozen storage (-10°C) the principal volatiles of parsley, myrcene, β-phellandrene and menthatriene were extensively degraded to non-volatile products at differential rates. p-Cymenene and the tentatively assigned menthatriene diepoxide were formed as minor volatile oxidation products. Myristicin remained largely unchanged. Under similar frozen storage, chlorophyll 'a' displayed significant degradation with only minor amounts of chlorophyllide 'a', pheophytin 'a' and 13^2 hydroxychlorophyll 'a' formed. Ascorbic acid was extensively degraded in timescales preceding monoterpene and chlorophyll loss. Thermal blanching of parsley extensively prevented the degradation of the monoterpenes, suggesting that endogenous enzymes were responsible for the changes. Elimination of oxygen, in the absence of blanching, prevented volatile loss, confirming the requirement for oxygen.

The hypothesis that peroxidase can operate in a co-oxidative couple with the flavonoid, apigenin-7-glucoside and hydrogen peroxide, as proposed by Yamauchi (1985), was investigated to establish its potential role in the degradation of terpenoids and chlorophyll. In model experiments, using horseradish peroxidase, menthatriene and chlorophyll showed extensive degradation only when all components of the couple were present. In addition the requirement for oxygen was also established. Naringenin and umbelliferone have been shown to behave similarly to apigenin, as co-substrates for peroxidase. Lycopene, with

some structural similarity to menthatriene, was also susceptible to co-oxidation. Polyphenol oxidase, proposed to operate in a similar fashion to peroxidase with mono- and di-phenols as substrates (Montedoro *et al.* 1995), in model experiments did not cause the degradation of chlorophyll. The co-oxidative role of lipoxygenase in parsley is believed to be of minor significance, however, it is likely to be responsible for the production of low levels of hexanal observed during thawing of frozen parsley.

From this thesis it is concluded that the aroma and colour quality loss in frozen unblanched parsley probably results from the oxidative degradation of the unsaturated monoterpenes and chlorophyll 'a' respectively via an oxidative cascade initiated by the action of peroxidase.

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Chapter 1

Introduction

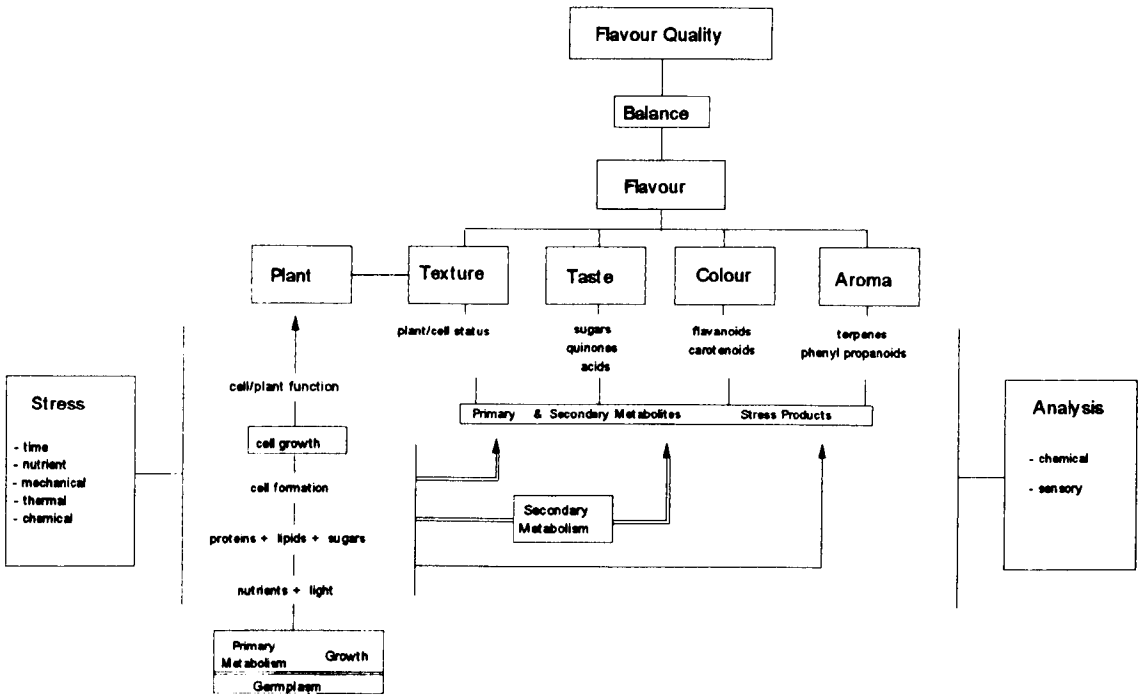
1.0 Introduction.

Over the last decade, the food industry in developed countries has progressed from a manufacture/production limiting industry to one increasingly driven by quality and choice. Food product quality or food acceptability has been aptly described as a marriage between food science and behavioural psychology (Thomson 1988) and represents a complex relationship central to the full exploitation of this area.

Flavour quality, a component of food product quality, combines human preference with sensory perception emanating from a range of biological sensors covering taste, colour and aroma. These sensory receptors are stimulated by a complex and specific range of

chemical species present in the food and are subject, through chemical and biochemical processes, to change and manipulation through raw material selection and the processing/storage regimes employed (Figure 1.1).

Figure 1.1 Flavour Quality in Vegetables



Within the natural products sector of vegetables and fruits, classical breeding studies have provided the germplasm for increasingly better quality produce. More recently, the developments in the field of molecular biology have opened up new avenues in the potential for tailoring germplasm to yield selective and desirable plant/fruit characteristics. Although the potential of this technology is formidable, an equally important parallel requirement is to understand the biochemical and chemical processes of the plant tissue relating to the key quality chemicals which are the stimuli for the sensory perception. Understanding of this type will identify the important enzymes which will need to be down-regulated or over-expressed to improve quality. Equally, the information is central to the more traditional approach to quality in the development/optimisation of the plant handling regimes employed, from agronomy through harvesting, processing and storage.

In this study, the focus is to establish the dynamics of the key quality chemicals as a

function of the processing stress applied and to identify the important chemical and biochemical processes responsible for any changes. These data will be utilized in proposing tailored technological processing conditions to improve quality. The approach employed in this study involves the controlled cultivation of plant tissue, the application of controlled post harvest tissue stress and the subsequent analysis of endogenous species using state of the art techniques designed to minimise artifactual change (Figure 1.1). Secondary experimentation is implemented to establish the respective roles of chemical and or biochemical processes and complemented with model protocols to challenge and extend the data generated.

The tissue selected for investigation in this study is the culinary herb, parsley, based on the relevance of reported storage stability data and sponsor interest.

In the following sections of this chapter, a detailed literature survey is presented, to establish the starting point for this study, within a structure aligned to establishing the chemical status of parsley as it is subject to typical commercial processing. Emphasis is subsequently placed on the type of processes responsible for change with particular focus towards endogenous enzymes.

This review was performed at the start of this study and reflects the information available at that time. Subsequent literature published within the study period is discussed in the Results sections in the respective chapters.

1.1 Information Sourcing and Collation

In recent times, the most effective route to scientific information is through on-line sources, utilizing host computers which offer structured access to many of the classical sources of information, be it periodicals, books, conference proceedings, government reports, patents, standards etc. (Chen 1987). Efficient access to on-line information is critically based on an effective search strategy tailored to the indexing system of a particular data base file. In this study five data base files have been utilized using two host computers, Dialog, Palo Alto, USA and Blaise-Line, British Library Bibliographic Service, London. Selection of the most appropriate file/s is clearly based on the type of

information required. In this study the search strategy covers three areas, agriculture, chemistry/biochemistry and food technology, which map the production of the tissue, biochemistry during growth and development and the harvesting, processing and human consumption. To cover these areas, three data base files have been used through the Dialog host:-

(i)	Biosis preview	File 5,55	1969- on (Biological Abstracts)
(ii)	CASearch	File 399	1967- on (Chemical Abstracts)
(iii)	FSTA	File 51	1969- on (Food Science & Tech Abstracts)

To design a search strategy, a series of questions about the information required need to be formulated, from which the key terms can be identified and converted into recognised terms, used by the indexing system of a particular file, and selectively combined using the logical operators.

(a) Search Question

What are the influence of endogenous enzymes on the flavour quality and shelf life of vegetables and herbs?

(b) Key Terms/Blocks

- vegetables and herbs	- tissue block
- flavour and quality	- flavour block
- enzymes	- enzyme block
- storage	- processing block
- flavour generation	- generation block

These terms can by expanded using the BIOSIS indexing language/structure, Dialog, Palo Alto, USA, (Appendix 1.1).

(c) BIOSYS strategy

Selective combination of the above terms using the logical operators (AND/OR) retrieves papers matching the strategy and these have been screened for suitability and retained in hard or soft copy form.

A similar approach was implemented for the CASEARCH and FSTA files plus the two main bibliographical files, BNBC (British National Bibliography) and LCC (Library of Congress) through the BLAISELINE host.

Besides retrieving relevant information from the host computers, it is also necessary to store retrieved data in a format that is easily accessible and can be searched further. To this end a "Programme Database, SB4PHD" has been established, using a 'Windows' based relational database package (SuperBase 4), which mirrors the structure of the programme discussed earlier through a tailored "user" indexing system. The record format, into which the information is transferred from the reference paper, consists of four components (i) source information, (ii) title information, (iii) indexing fields and (iv) usefulness rating fields (Figure 1.2).

Figure 1.2 Literature Database Record Format

Reference Number:	1	Relevance Rating: ***	Report Flag: P
Title:	Storage time temperature relationships and stability of chlorophylls, colour and total flavour emmission in frozen parsley		
Authors:	Philippon J; Rouet-mayer M; Fontenay P; Duminil J		
Source:	Sci Aliments		
Year Volume Page:	86 6 (3) 433-46	book status (K,I)	
Book Reference:			
Scientific Category:	Empirical	Na	
Tissue/Product Type:	Parsley		
Chemical Category:	Volatile	Chlorophyll	
Processing Stress:	Frozen	Storage	
Quality Category:	Colour		
Method Category:	Na		
Additional Keywords:	discolouration volatile-loss		

The key to the success of the system is the effectiveness of the indexing fields, in the "SB4PHD" system where six indexing vectors are utilized, namely, tissue type, chemical category x2, processing stress x2, quality type, analytical method type and science category x2. Associated to each of these fields are a limited set of keywords which allow,

in combination, the context of the paper to be described (Table 1.1).

Table 1.1 Programme Database Indexing Keywords

Indexing Field	Keywords		
Tissue	Parsley, Parsley Cell Culture, Herb, Vegetable, Plant, Fruit, Green Beans, Tomato, Potato, Model, Non Tissue, Wine, Tea, Microorganism, Animal.		
Chemical Category	Ascorbic Acid, Phenolic, Coumarin, Flavanoid, Carotenoid, Chlorophyll, Enzyme, Lipid, Volatile, Protein, Carbohydrate, Ice, Water, Oxygen, Inorganic, Non Volatile.		
Processing/Stress	Chemical, Dehydration, Fermentation, Frozen, Gas Atmosphere, Irradiation, Mechanical, Packaging, Preharvest, Storage, Thermal.		
Quality	Aroma, Colour, Taste, Texture, Quality.		
Method	Extraction, Separation, Purification, Activity, Colour, Texture, Sensory, Analysis, Genetic.		
Science	Biology-Agronomy Biology-Genetics Biology-Histology Biology-Physiology	Chemistry-Biological Chemistry-Compositional Chemistry-Inorganic Chemistry-Organic Chemistry-Physical	Physics-Mechanics Physics-Radiation Engineering

Each paper is reviewed, indexed and the two 'usefulness' fields set accordingly. For retrieval purposes all of the field's 'text strings' are searchable, singly or in combination. However, information is generally retrieved using the indexing system. For example, 44 papers are retrieved using the search terms, parsley (tissue field) AND volatiles (chemical category field; Appendix 1.2). To date, 866 papers have been referenced using this system. Utilization of this system in the design of this study and the preparation of this document has been invaluable.

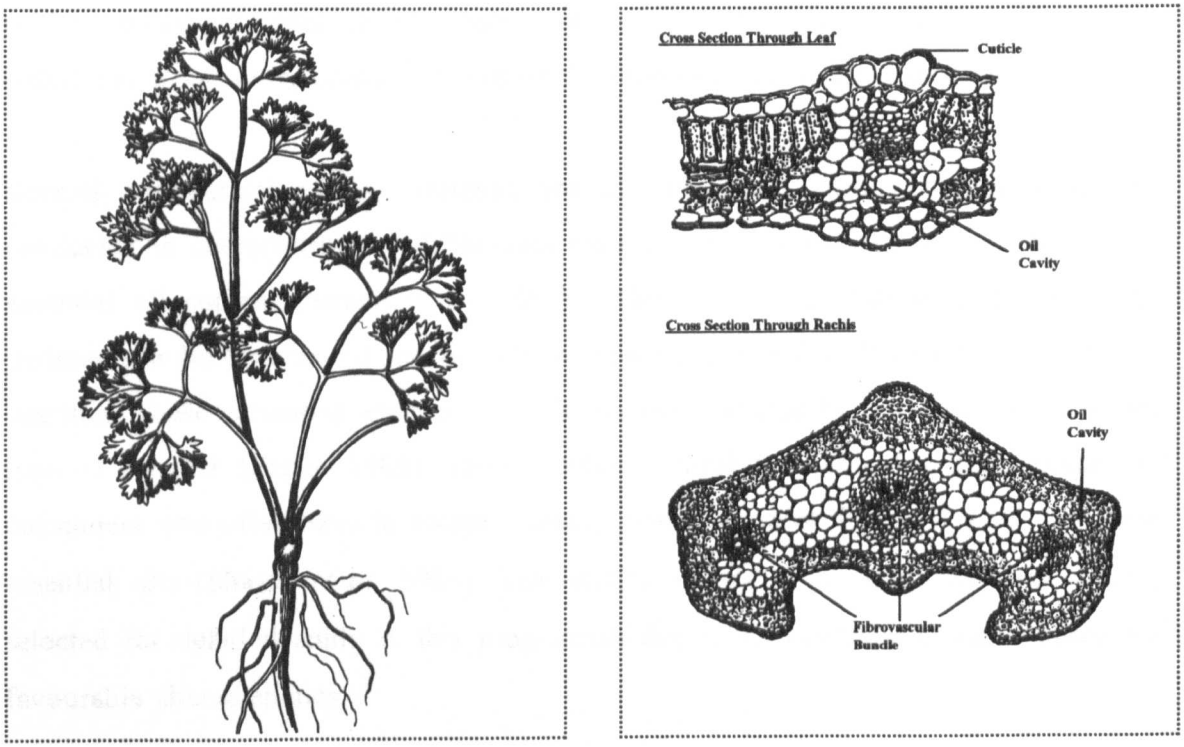
1.2 Parsley, the Culinary Herb

Parsley, [*Petroselinum crispum* (Miller) Nyman ex A.W.Hill, *Umbelliferaceae*. (syn *Petroselinum sativum*, *Petroselinum hortense*, *Apium petroselinum* (Uphof 1968)], is

probably native to Southern Europe. However, so long has been its cultivation, that the original distribution is uncertain and may include Eastern Europe and Western Asia, (Page *et al.* 1980).

Parsley is a biennial herbaceous plant which grows to a height of approximately 30 cm. In the first year the foliage is finer and more tender than in the second year when preparing to flower and seed and therefore tends to be commercially grown as an annual for culinary purposes. The histology of the parsley plant reveals the presence, in both leaflets and rachis/petioles, of oil cavities and ducts (Parry 1969), in which the essential oil of the plant is believed to accumulate (Rouet-Mayer *et al.* 1986; Figure 1.3).

Figure 1.3 ***Petroselinum Crispum***



There are two major types of cultivated parsley, common or curly leaved varieties eg. moss curled, used for its leaves, and plain or flat leaved varieties eg. neopolitanum (or Italian parsley) used both for its leaves and roots (Simon *et al.* 1988). A third lesser cultivated variety, the so called Hamburg parsley (var *tuberosum*) is used for its swollen root parts as a vegetable.

Today, parsley is cultivated mainly in USA, UK, Germany, Dominican Republic, Mexico, Egypt, Hungary and Yugoslavia but production information is limited. The major markets for parsley are USA, Germany, UK and France (Greenhaugh 1979; Dumville 1988; Maftei 1992). Commercial utilization of parsley takes two primary forms. Firstly the leaves and stems are used as a garnish or flavouring aid in soups, vegetables, salads and sauces marketed in either the fresh, dried or frozen state. Alternatively parsley is used to obtain the essential oil where seed oil, herb oil or leaf oil are used in the flavouring and fragrance industries. The origin of the seed and leaf oils is self explanatory, herb oil results from the steam distillation of all aerial parts of the plant including the immature seed heads (Porter 1989). In terms of the relative oil yields, seed yields most oil (1.3-3.5 % fresh basis; Porter *et al.* 1985; Kim *et al.* 1990) followed by herb oil and leaf oil (0.04-0.15 % fresh basis; Simon *et al.* 1986,1988). However, the quality of the oil, based on its similarity to characteristic parsley aroma from fresh tissue, follows the reverse order. These properties combine to dictate the relative commercial cost of these materials.

Several commercial parsley varieties are available and various studies have been conducted to characterize the differences between them, especially in relation to their essential oil content (Simon *et al.* 1988). The general conclusion is that there are variations in the essential oil constituents and levels and therefore there are diverse flavour qualities. These variations, caused in part by variety, but also by growing conditions and time of harvest (Porter 1989), cause quality control problems for the essential oil consumers who often have to accept a much greater variation for parsley oil than other essential oils (Shaath *et al.* 1988). The parsley variety "Smaragd" has been initially selected for detailed study in this programme due to its commercial significance and favourable characteristics.

1.3 Parsley Cultivation and Commercial Processing

In this study the focus is on the use of parsley tissue as a food ingredient rather than as a raw material for essential oil production. However relevant information from the latter will be used to support the former activity.

Commercial parsley is largely field grown. It is typically sown in rows approximately 0.7m apart with approximately 30 plants per metre. Seeds are sown in well worked moisture-retaining soil in April and take about 3 weeks to germinate. For garnishing/direct flavouring purposes, where parsley leaf material is incorporated within a product or marketed directly to the consumer, harvesting of the whole plant takes place approximately 12 weeks after germination/emergence. For essential oil production, harvest is dictated by the source of the oil ie leaf or seed and thus the development stages of the crop ie immature seed head/mature seeds, determines the time of harvest. The exact point of harvest for tissue or essential oil purposes is often dependent on following some endogenous indicator such as vitamin C or essential oil content; often, early morning is the preferred harvest time.

After harvesting, subsequent processing is clearly dependent on the means of preservation employed (ie fresh, dried or frozen) and the final form the product is to take. Generally, after harvesting, the plant is washed to reduce the microflora load (Kaeferstein 1976) and centrifuged to remove excess water (Koslowski 1979). Under fresh storage, parsley is generally packaged in air tight polymer films often with modified gas atmosphere at chill temperatures (0-5°C) where shelf-lives of 4-8 weeks are typical with extension to 12-16 weeks if disease free young leaves are used (Apeland 1971). In the absence of these protocols, fresh parsley has a shelf life of approximately 3-7 days (Wills *et al.* 1984). For dehydrated parsley, drying conditions typically involve active ventilation at temperatures not in excess of 100°F (38°C) under reduced light, conditions designed to minimise the decline of essential oil content during processing and provide a product shelf-lives of 1-2 years (Splittstoesser 1984).

Finally, frozen storage typically excludes any pre-freezing thermal blanching process due to the flavour loss associated with blanching (Koslowski 1979). Freezing rates tend to be rapid and the final product is stored in bulk at temperatures as low as possible (< -25°C). In all the above treatments some form of mechanical leaf/stem separation process will be employed prior to final packaging and distribution.

1.4 Quality Status of Parsley During Processing and Storage

Papers reviewed in this section are retrieved using the indexing fields, tissue= parsley AND quality=aroma-texture.

In this section the quality issues associated with commercially marketed parsley tissue are reviewed to provide a focus for subsequent chemical/biochemical studies. It is appropriate to sub divide the section into the three major forms by which parsley is marketed, namely fresh, dried and frozen, and to highlight the different quality aspects within these classes. The most important quality factor for fresh parsley is the freshness/turgor of the sprig followed closely by the colour (Apeland 1971; Ryall *et al.* 1972) and the aroma/taste. Clearly the first two components have a visual basis and largely influence the consumer's initial perception and potentially mediate subsequent stimuli (eg aroma/taste, mouthfeel). These characteristics are relevant to parsley in a low or medium state of division (ie sprigs, leaflets). Under conditions of increased division, turgor would/could not be assessed and colour and aroma/taste would be of primary importance.

Fresh parsley is probably the most utilized form (Halva 1985) and is subject to a number of quality issues dependent on how it is handled and the duration of storage. After harvesting, loss of turgor (ie wilting) occurs rapidly, due to water loss, unless the tissue is stored under elevated humidity (around 90%) and or cooled (0-5°C) (Wills *et al.* 1984; Ryall *et al.* 1972). Additionally, like other fresh herbs, parsley is susceptible to leaf yellowing and decay on storage. This quality loss can be retarded by using modified packaging atmospheres (Aharoni *et al.* 1989). The loss of aroma volatiles does not appear to be a quality issue in fresh parsley as reported below for other preservation methods, probably because of the relatively short storage times and the predominance of other quality attributes. However, under certain gas atmospheres, anaerobic respiration gives rise to off flavours (Aharoni *et al.* 1989). In addition to the above factors, parsley quality is also dependent on the maturity of the leaves as young leaves exhibit lower respiration rates thus delaying senescence and extending storage life (Apeland 1971).

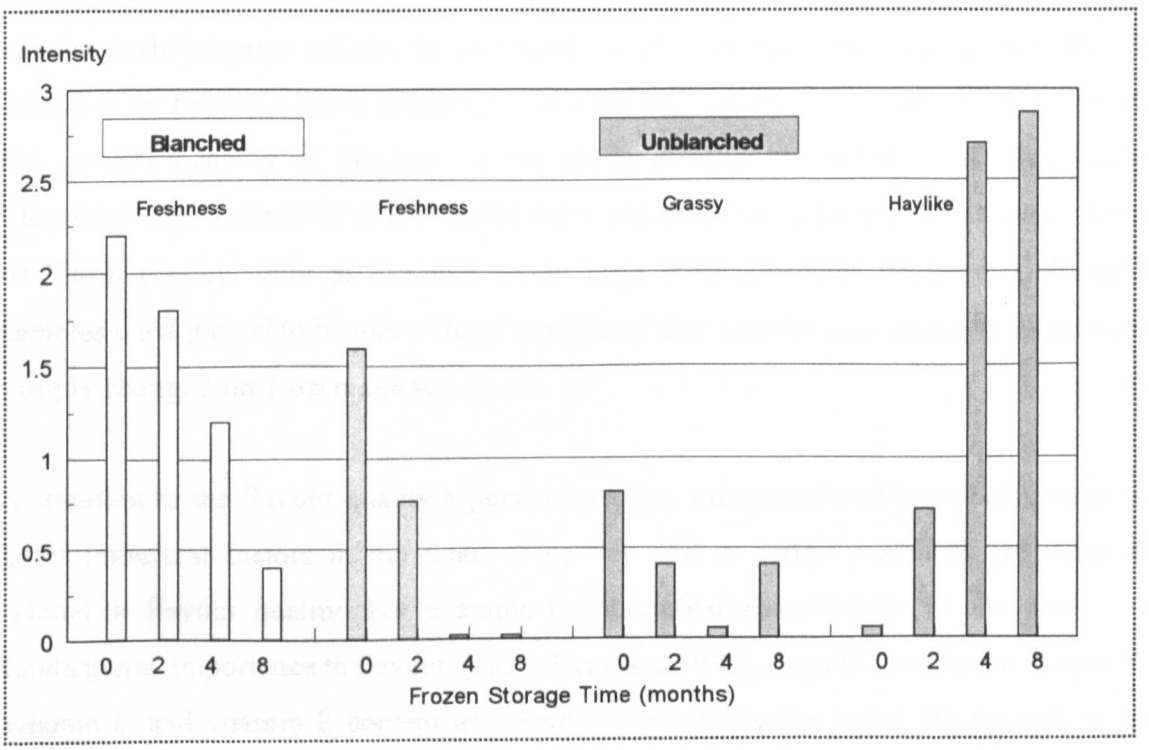
Dried parsley is another important form of the herb. Here the state of division of the leaves tends to be greater than fresh or frozen and the consumer assessment and

expectance of turgor is therefore not the major quality attribute as discussed earlier, being superseded by colour and aroma/taste. The thermal processing involved in drying parsley causes a loss of green colour and a reduction in the herb aroma quality, the latter being related to the exposure to air and light during the process. On storage, dried parsley displays a general yellowing of leaves occurring over a period of 1-2 years (Berset 1983). The German standard (Dried Parsley 1980) describes the commercial quality criteria for this herb.

Preservation of parsley by freezing offers a number of potential advantages over the modes of preservation given above, in that the inherent disadvantages of these methods, related either to volatile loss during thermal dehydration or rapid senescence under chilled storage conditions, do not immediately apply. For example frozen parsley has a much more highly developed aromatic quality compared to dried parsley, and this is one of the reasons that manufacturers of prepared frozen meals increasingly use frozen parsley (Philippon 1986). However, despite its advantages, frozen parsley is subject to a number of quality loss phenomena dependant on the exact processing regime employed. For example, storage temperature is particularly important, with shelf-lives ranging from days at -8°C, (Philippon *et al.* 1986) to 1 year at -24°C (Duden 1981). The process of freezing and thawing of parsley, like many other leafy vegetables, causes tissue damage due to ice crystal formation and gives the leaves a water soaked/darker appearance with a loss of texture (Ryall *et al.* 1972) and these authors classified parsley as being 'moderately susceptible' to freezing injury compared to other vegetable tissues. Additionally, colour changes have been reported for parsley under frozen storage (Philippon *et al.* 1986), and discussed in terms of a reduction in the "high quality storage life". However the actual colour changes are not recorded, merely related to chlorophyll loss. Typically, colour changes in vegetables under frozen storage involve a loss of the fresh/bright green appearance with the dominance of olive green/brown coloration (Desrosier *et al.* 1977). The final type of quality loss in frozen parsley relates to aroma/taste changes. Two categories have been reported, involving the general loss of aroma character (Philippon *et al.* 1986), and the formation of off-flavour. The subject of off-flavour generation and the role of enzymic degradation of polar lipids in frozen parsley has been extensively studied by Duden (Duden 1984, 1985) and coworkers (Scholz 1982; Fricker 1981; Hubner 1981), and offers an important contribution to the starting position of this programme. In

their study, they performed a comprehensive series of sensory experiments recording both taste (odour/taste) and odour (smell) for two varieties of parsley, flat and curly leaf, as a function of storage time/temperature with and without blanching (Duden 1984). The experimental protocol used, involved removing the large stems from freshly harvested material, washing and gentle centrifugation. Samples for blanching were immersed in boiling water for 30 seconds and subsequently cooled in a cold water wash. All material was frozen at -50°C in flat layers in plastic bags and transferred to the test temperature (-50°C, -24°C, -18°C, -12°C) for storage (0, 2, 4, 8 months). Prior to sensory assessment, material was ground at -18°C and allowed to thaw for 20 minutes at room temperature. From their data, the scores for 'odour only' assessment followed those of taste and only the latter were presented. Two attributes were highlighted, firstly 'freshness' which was a positive attribute and secondly (as a negative component), 'off flavour' scored as 'grassy' and 'hay like'. Figure 1.4 shows the 'freshness' scores for curly leaf parsley stored at -12°C over a period of 8 months, for blanched and unblanched material. From these data they concluded that blanched samples were superior to unblanched.

Figure 1.4 Sensory Descriptive Analysis for Frozen Parsley (-12°C) (Duden *et al* 1984)



However this conflicts with typical commercial practice where material is unblanched, due to the extensive loss of aroma and colour quality. Figure 1.4 also compares the temporal profile for the attributes 'grassy' and 'hay like' at -12°C for unblanched parsley. Interestingly the 'grassy' character is shown to be most intense in unstored material and decays with storage whilst the 'hay like' character is not present in freshly stored material and develops, to cause significant quality loss after 6 months and longer. Equally interesting is their record that the 'grassy' character only occurs in unblanched material and that the 'hay like' component although present in both is more pronounced in similarly unblanched tissue. Finally, the effect of freezer temperature after 8 months storage on the 'grassy' and 'hay like' attributes showed the 'hay like' score to increase consistently with temperature however the 'grassy' attribute remains unchanged at -18°C and below, with a reduction at only -12°C and may offer some information on the type of process responsible, be it chemical or biochemical. Fish- like off flavours are also reported in stored frozen parsley (Duden 1981).

To date, the quality/sensory issues relating to aroma have been the primary focus in stored parsley. For colour, however, literature tends to include chlorophyll measurement in concert with visual assessment, perhaps due to an established correlation between the two, and as such is more effectively reviewed further in the following section. Finally, literature on taste as a separate category is scant and typically assessed with aroma under the general heading of flavour. In the study referred to earlier by Duden (1984), 'sharpness' and 'bitterness' at low levels were perceived as positive attributes and typical of 'fresh' parsley; only at elevated levels were these attributes negative. Unblanched samples were judged to be more 'sharp' and 'bitter' than blanched samples although neither sample changed on frozen storage.

In addition to the flavour quality aspects of parsley, nutritional and microbial quality are other important factors in the retail of parsley and in certain instances are indirectly related to flavour quality. For example the microbial/disease status of the plant is of fundamental importance to flavour quality (Apeland 1971). Equally nutritional factors like vitamin C and vitamin E content and their relationship to the redox status/capacity and antioxidative capacity respectively, of the tissue may serve to influence flavour quality.

From this review, the increasing relative importance of frozen parsley as a mode of preserving the quality of this herb was highlighted. Although a number of quality issues are still apparent, the potential advantages for this preservation mode suggest that it is a sensible focus for this study. Clearly, understanding from the other modes of preservation will be applied, particularly for fresh parsley, as in both cases, living plant tissue is involved and many of the processes of senescence will be relevant.

1.5 Chemical Status of Parsley During Processing and Storage

In this section the chemical status of parsley during processing and storage is discussed. It is important to relate this information to the quality status, in order to establish the relevance of the information and the usefulness of using a chemical quality indicator in tailoring the processing regime to quality ends. This section is arranged with this emphasis and considers chemical classes which are empirically linked to quality, eg volatiles with aroma , chlorophyll with colour etc. The reader is refered to Chapter 3 'Development of Analytical Methods' for comparative discussion on the technique presented here.

1.5.1 Volatiles (Aroma).

Papers reviewed in this section are retrieved from the 'Programme Database', using the indexing fields, tissue= parsley AND chemical class=volatile AND processing=all.

From a flavour perspective, volatiles are low molecular weight organic compounds which have sufficiently high vapour pressure and odour threshold/intensity to be organoleptically significant, singly or in combination.

To date literature on volatile flavour analysis of parsley has been split largely into three areas :-

- identification of flavour components.
- studies on the generation of flavour
- effect of stress on flavour components/levels.

Identification studies have largely been associated with essential oil production/characterization, which by definition utilizes a steam distillation extraction

process. This process involves division of the tissue prior to steam reflux and is a type of total flavour analysis utilizing multiple extraction of the sample with steam coupled to co-condensation and partitioning into an organic solvent which can be subsequently analysed by GLC. The main problem with the method, as far as the volatile composition of parsley tissue *in vivo* is concerned, is that the thermal, mechanical and chemical extraction environments are severe compared to the mild conditions found during commercial fresh or frozen production and certain caution must be applied when viewing the results. This caution is well-illustrated by the work of Fischer *et al.* (1987) who showed that the extraction methods applied to Marjoram produced major differences in the monoterpenes profiles which in the case of steam distillation, resulted from hydrolysis reactions (see Section 1.6.1). Although steam distillation may not be ideal, it is a rich source of information on the types and amounts of aroma components in food materials. The most comprehensive papers on parsley are from Kim *et al.* (1990), Shaath *et al.* (1988), and MacLeod *et al.* (1985) although the contributions of earlier works by Garnero *et al.* (1968), Kasting *et al.* (1972) and Vernon *et al.* (1983) should also be recorded. More recently, activities have been reported which have been focused towards the quality of parsley tissue and thus include the important organoleptic assessment as well as using analysis regimes which are more consistent with the ideal analysis system discussed later. An excellent paper by Jung *et al.* (1992) utilizes 'aroma extract dilution analysis', AEDA, to identify the flavour components which are of primary olfactory significance in parsley leaves. Jung *et al.* cite fourteen odour components with high dilution factors, of which six are newly identified (Table 1.2). Examination of the components in Table 1.2 reveals the notable absence of C6 aldehydes and alcohols produced enzymically/chemically from unsaturated lipids (see Section 1.6.). In view of the odour threshold of these components and their reported concentrations, see later text, this is initially surprising. Clearly an explanation may simply be that they are, below the AEDA threshold applied in the generation of the table, or the relevance of the concentration data. However, examination of the extraction procedure employed by Jung *et al.* (1992) shows that enzymic processes are likely to be minimised under the low temperatures and methanol content and thus the components identified may not reflect all the normal generation processes available under standard usage of parsley. In earlier studies on the generation of flavour, the headspace of macerated parsley leaves in the presence and absence of enzyme inhibitor was investigated and lipoxygenase type products were noted under an inhibition regime

suggesting that autoxidation processes are operative within the analysis protocol (Freeman *et al.* 1975). Future work will be required to establish, when and under what conditions, C6 components are formed and at what levels. Berger *et al.* (1985) investigated the important odour components in pineapple and identified a series of undecenes with ultra low detection thresholds. Application of the methodology to parsley revealed the presence of 1-(EZ)-3,5-undecatriene under a regime of enzyme inhibition.

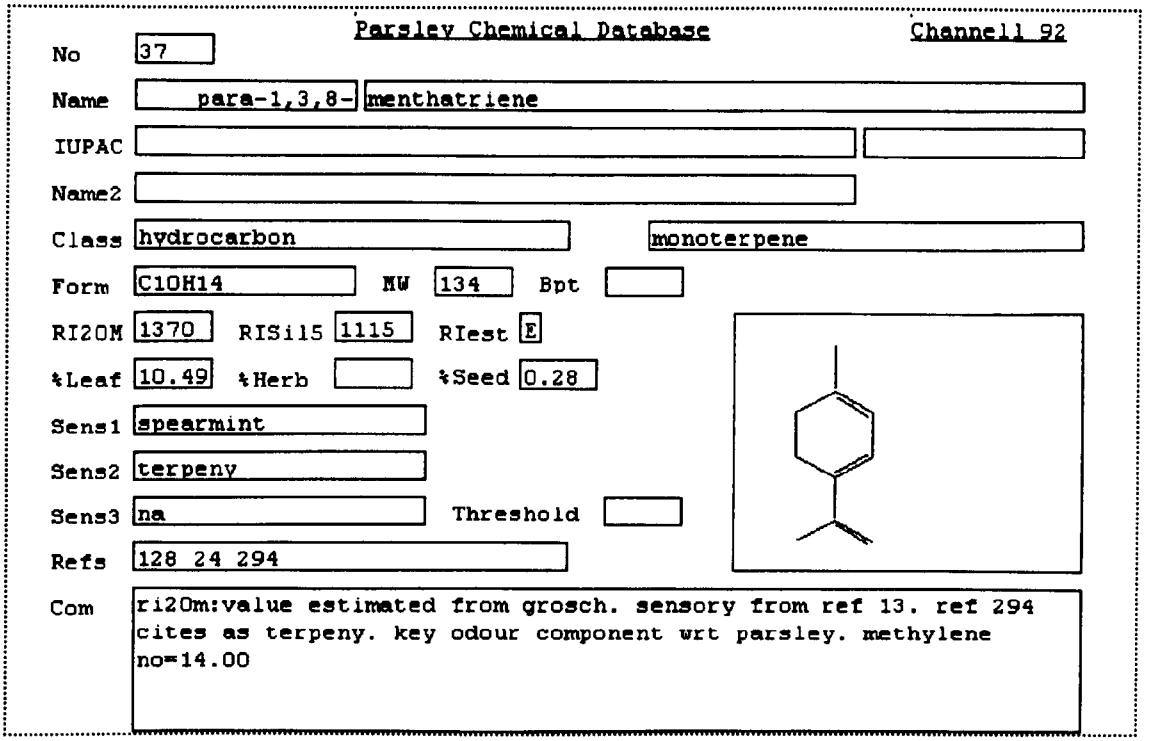
Table 1.2 Potent Odourants of Parsley (Jung *et al.* 1992).

Flavour Group	Flavour Component	Odour Description
II	2-isopropyl-3-methoxypyrazine	musty
	2-sec-butyl-3-methoxypyrazine	musty
	methyl 2 methylbutanoate	fruity
	myristicin	spicy, nutmeg-like
	p-mentha-1,3,8-triene	terpeny
	β-citronellol	rose-like
	linalool	floral
	myrcene	herbaceous, metallic
	β-ionone	violet-like
	(E,E)-2,4-decadienal	fatty
	1-octen-3-one	mushroom-like
	(Z)-1,5-octadien-3-one	geranium-like
	(E)-6-decenal	cucumber-like
	Unknown	musty, woody

To collate all the flavour chemicals cited in the above references, a computer data base was designed, using a 'Windows' based relational system (SuperBase4). For each compound cited, data on the physical, chemical and sensory properties including chemical structure, sensory threshold, retention indices and the amount of each compound in tissue was entered (Figure 1.5). Selection for inclusion into the database was largely as a function of volatility, however increasingly higher molecular weight compounds are being identified, through the application of more sensitive techniques, and reported to have sensory importance though either aroma or taste modalities, for example the phthalates,

coumarins and furocoumarins (Nitz *et al.* 1991). Selective inclusion of these chemicals has also been undertaken. The system was established as a tool to support the experimental and interpretative components of this study, for example, the retention indices on polar and non polar phases to aid identification, chemical structure for chemical reaction considerations and sensory descriptors for quality aspects.

Figure 1.5 Chemicals Database Record Format



For completeness, certain of the indexed fields have been estimated when specific information was unavailable, eg. retention time. Equally the subject of sensory descriptors and threshold data is an extremely complex area, due to the descriptive language employed and the various protocols used to obtain threshold information. In this database the descriptors used largely originate from Artcanter (1969), unless more recent specific GC/olfactory data are available. Threshold data originate from the compilation of standardized values from Devos *et al.* (1990) with support from earlier works (Stahl 1973, Fazzalari 1978). When compared to the TNO compilation study of Maarse *et al.* (1989), the compilation was found to be comprehensive with the addition of the more recent works. Appendix 1.3 provides a full listing of all the chemicals recorded, sorted alphabetically on the common or trivial name.

Table 1.3 ranks the components by their percentage composition in the parsley leaf as reported by Kim *et al.* (1990).

Table 1.3 Chemical Database Sorted by Percentage Leaf Composition

Chemical Name		Chemical Class		Emprical Formula	mw	Thes hold	% Seed Level	%[2] Leaf Level
	myristicin	phenyl prop[1]	methoxy	C11H12O3	192		47.54	21.80
para-1,3,8	menthatriene	monoterpene	hydrocarbon	C10H14	134		0.28	10.49
beta-	phellandrene	monoterpene	hydrocarbon	C10H16	136		7.52	9.96
alpha-	terpinoline	monoterpene	hydrocarbon	C10H16	136		0.14	5.51
	unknown	unknown	unknown					4.57
4-iso-	propenyl methyl benzene	monoterpene	hydrocarbon	C10H12	132		0.28	4.52
cis-3-	hexen-1-ol	aliphatic	alcohol	C6H12O1	100			3.10
para-	menthatrienol	monoterpene	alcohol	C10H14O1	150			3.05
	elemicin	phenyl prop	methoxy	C12H16O3	208		2.64	3.04
	myrcene	monoterpene	hydrocarbon	C10H16	136		0.54	3.01
	unknown	unknown	unknown					2.77
	limonene	monoterpene	hydrocarbon	C10H16	136	5.61	0.10	2.63
trans-2-	hexenal	aliphatic	aldehyde	C6H10O1	98	6.88		2.08
	unknown	unknown	unknown					1.94
alpha-	copaene	sesquiterpene	hydrocarbon	C15H24	204			1.32
	germacrene d	sesquiterpene	hydrocarbon	C15H24	204			1.15
beta-	elemene	sesquiterpene	hydrocarbon	C15H24	204			0.90
	bicyclogermacrene	sesquiterpene	hydrocarbon	C15H24	204			0.86
alpha-	pinene	monoterpene	hydrocarbon	C10H16	136	5.41	22.28	0.83
	unknown	unknown	unknown					0.82
alpha-	gurjunene	sesquiterpene	hydrocarbon	C15H24	204			0.82
para-	cymene-8-ol	monoterpene	alcohol	C10H14O1	150		0.09	0.73
alpha-	phellandrene	monoterpene	hydrocarbon	C10H16	136		0.28	0.72
l-	carveol	monoterpene	alcohol	C10H16O1	152			0.65
beta-	farnesene	sesquiterpene	hydrocarbon	C15H24	204		0.08	0.65
	tetradecanal	aliphatic	aldehyde	C14H28O1	212			0.64
para-	methyl acetophenone	aromatic	ketone	C9H10O1	134	7.43	0.10	0.61

1. Phenyl Propenoid

2. Data from Kim *et al* (1990)

The second group of studies on parsley involve following flavour volatiles as a function of various processing stresses. This section is analogous to the earlier quality section except quality/sensory measurement is replaced by instrumental measurement and

therefore allows potential correlation between these two key parameters.

Our term "processing stress" can be categorized into:

Preharvest stress:	nutrient stress
	climatic stress
Post-harvest stress:	thermal stress (heat,frozen)
	mechanical stress (cutting, bruising)
	chemical stress (gas atmosphere, additives).

Although preharvest stress can have a significant influence on flavour, as with other secondary products (Bernath 1986), the focus of this study is primarily towards postharvest stress. The only proviso being that any control variables which may exist at the point of harvest would be considered in any "product specific treatment" development. Such variables may in fact determine the time of harvest, for example vitamin C (Zderkiewicz *et al.* 1972), essential oil (Porter 1989) and chlorophyll level.

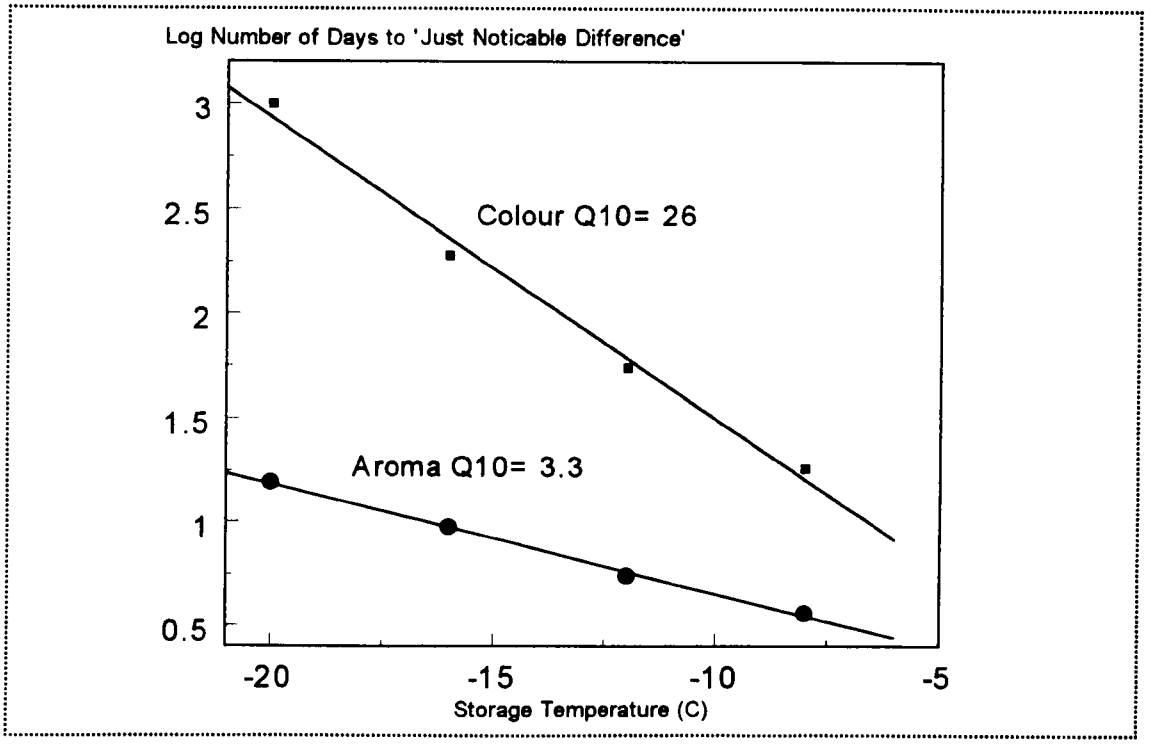
The effect of post harvest stress on parsley volatiles has largely been researched with frozen parsley in mind, and particularly by a group of workers at the Centre National de la Recherche Scientifique laboratory, CNRS, (Rouet-Mayer *et al.*/Phillipon *et al.*) who in 1986 were involved in a broad programme intended to define the conditions for obtaining 'more highly developed aromatic quality' than typically available from air-dried parsley. It is interesting to note that under drying conditions, not only is two thirds of the volatile oil lost but the remaining oil has appreciably the same composition (Vernon *et al.* 1978). In the two papers from this group (Philippon *et al.* 1986; Rouet-Mayer *et al.* 1986), the first quantifies (i) total headspace change using a non qualitative technique (Rouet-Mayer *et al.* 1983) and (ii) chlorophyll change, under a storage time/temperature regime with the aim to calculate the 'temperature quotient, Q10' often quoted for frozen vegetables/foods. This value is defined as the ratio of the rate of change of a property or attribute at one temperature to the rate of change at a temperature 10°C higher or lower. A slightly more usable form of the definition is the ratio of the time required to bring about a specific change (ie just noticeable difference, JND) at one temperature, to the time required at a temperature 10°C higher. The basic experimental design of the group involved storing unblanched parsley at five storage temperatures (-8°C, -12°C, -16°C, -20°C and -65°C / control) and performing chemical and sensory analysis as a function of

time (0 to 170 days). Initially the group quantified the changes in total headspace which equated to a 'just noticeable sensory difference' based on 11 out of 15 panellists recording a correct pairing in a triangle similarity test ($0.01 > P > 0.001$) between a control sample (stored at -65°C and showing no change) and a sample showing reduced total headspace values. The point of this JND equated to a 23% reduction in the total headspace. The data for total headspace versus time followed a hyperbolic function as shown by the plot of $(\text{total headspace})^{-1}$ versus days. Interestingly this linear representation reveals a biphasic plot with a transition time around 26 days for all the temperatures. Although the authors recognise the possibility of the existence of two separate processes, the basis is unidentified. However reference is made to a potential contribution due to the permeability of the packaging material (polyethylene) and the pack size. Nitz *et al.* (1989) also cited rapid deterioration in the organoleptically important component, menthatriene, on frozen storage and showed the process to be accompanied by an increase in two, to date, unreported oxygenated reaction products each with their own organoleptic character namely, an endoperoxide, described as fruity/sweet and a diepoxide with an unpleasant/sweaty character. For this study, the data presented by Philippon *et al.* (1986) is very useful as it provides a potential starting point for our studies and is consistent with our general philosophy of controlling stress and measuring the chemical/biochemical status. The only development that we will implement is to introduce a qualitative component to the chromatographic analysis and to control stress to a much greater degree, such that the processes that are operative can be identified. From Philippon's plot of $(\text{total headspace})^{-1}$ vs time (days), the Q10 value can be determined by reading from the graph at a horizontal transect equivalent to the JND value (23% of the control), the number of days at the four temperatures studied. Using these data, Figure 1.6 shows the standard plot of log days versus temperature where, from the slope, the Q10 value of 3.3 for aroma is obtained.

Significantly, the linear form of this plot suggests parsley is consistent with the laws governing other vegetables and fruit. However, the value is particularly small compared to say green beans, Q10=8.1, indicating that parsley is less sensitive to change. However, if the absolute rate of change (time to JND at standard temperature, -18°C) of the two are considered, as it should be, green beans score 300 days whilst the much more labile system of parsley scores 11 days.

Rouet-Mayer *et al.* (1986) also considered processing stresses relevant to frozen parsley. In investigating the effect of blanching they measured total headspace as a function of blanch time (0, 5, 15, 30, 60 and 90 sec at 100°C) at three subsequent storage conditions (i) unstored, (ii) stored for 35 days at -65°C (iii) -12°C, monitoring peroxidase activity (with guaiacol as substrate) immediately after blanching.

Figure 1.6 Temperature Quotient Plot for Frozen Parsley



From their data, a clear and significant loss of volatiles resulted directly from the thermal blanching process for all samples. No additional losses were recorded for the -65°C stored sample over the unstored sample, however, in sharp contrast, samples with blanch times below 30 seconds and stored at -12°C showed major additional loss, (~64% @ 0 sec), whilst above 30 seconds no additional loss occurred. The authors conclusion was that enzymic processes contribute to aroma loss in frozen parsley although no work on enzyme or substrate identification was performed. The effect of permeability of packaging material, polyethylene versus polyester, was also studied and shown to have a direct relationship with the disappearance of volatiles. It would, however, be interesting to establish whether this is due to evaporative loss, as supposed, or perhaps oxidative change due to oxygen ingression, as discussed later.

The final stress considered was the effect of tissue grinding, performed in the frozen state (-20°C), prior to storage (32 days). At the two temperatures studied, -65°C and -12°C, no change in total headspace or, more interestingly, sensory quality was observed. Grinding increases cell disruption and might have been expected to produce a quality change resulting from chemical and biochemical processes initiated by reactant mixing, and the activation of lipoxygenase and other stress enzymes. The implication here is that such processes do not occur or the action of freezing effectively normalises the tissue prior to grinding. Certainly freeze/thaw regimes are effectively employed as a means of cell disruption and are used in the biotechnology industry (Keshavarz *et al* 1987).

To date we have almost exclusively concentrated on stresses related to frozen parsley, however it is worth briefly reviewing some of the interesting stress effects recorded for other forms of parsley. In particular the work of Deans *et al.* (1991) who compared the effect of conventional warm air drying with microwave drying of herbs. Under conventional drying, similar volatile loss was observed to that mentioned above ie reduction in total amounts with no new peaks. However under a microwave regime very large and significant new peaks were formed, with parsley registering the largest of those studied, accounting for 33.6% of the volatiles. All herbs studied (except savoy) showed the formation of new peaks. The other interesting observation from this study was the degree of volatile loss under conventional drying, where Rosemary stood out with regard to volatile oil retention. From the work of Pizzocaro *et al.* (1985), Rosemary was shown to have an antioxidative environment with the active species established as rosmaridiphenol, whilst parsley was the only pro-oxidative environment. This seems to suggest that although evaporative loss plays an important role in air drying, perhaps oxidative changes are also significant and may also be applicable to lower temperature work. This concept of chemical oxidative stress will be considered further in Section 1.6.

1.5.2 Pigments (Colour)

The main pigment classes within vegetables and fruits are the green chlorophylls, the yellow to red carotenoids and the red to violet anthocyanins. In green photosynthetic leaf tissue, the chlorophylls and carotenoids predominate with the former dominating visual appearance.

(i) **Chlorophyll**

The papers reviewed in this section have been retrieved using the indexing fields: tissue=parsley AND chemical class 1&2 =chlorophyll.

Chemically, chlorophyll consists of a magnesium containing porphyrin ring with a C_{20} phytyl hydrocarbon side chain. Chorophyll is located in the chloroplast of higher plants and forms a pivotal component of the photosynthetic apparatus. Parsley has one of the highest levels of chorophyll pigments of all vegetables (Gross 1991), containing 198.2 mg/chlorophyll 100g fresh wt (Yamauhi 1980) and is matched by a proportionately high level of carotenoids (Heinonen *et al.* 1989) and ascorbic acid (152mg/100g fresh wt; Wills *et al.* 1984).

In a parallel study to the work performed on volatiles, Philippon *et al.* (1986) performed sensory evaluation of colour and chemical analysis of chlorophyll during the frozen storage of parsley at a range of temperatures. From sensory triangle tests the panel registered a 'Just Noticeable Difference' (JND) equivalent to a 20% loss in the level of chlorophyll. The rate of loss of chlorophyll is shown to be a first order process with the greatest change at -8°C and virtually no change at -20°C. Application of the JND values to these data allows a temperature quotient of Q10=26 to be determined (Figure 1.6). In comparison with the data presented earlier for aroma, Q10=3.3, colour change is clearly considerably more sensitive to frozen storage temperature. However the absolute time for a just noticeable difference at typical frozen storage temperatures (-20°C) is substantially longer (800 days vs 16 days respectively). This is shown by the form of converging plots (Figure 1.6) suggesting that, by extrapolation, aroma and colour have comparable JND values at temperatures just below zero. However, at typical frozen storage temperature, 'high quality' loss will be caused by aroma long before colour comes into play. In fresh parsley stored at 1°C and 20°C, similar chlorophyll loss is observed with 15% and 65% reduction in 5 days respectively (Yamauchi 1980). In a study of the effect of modified atmospheres on a range of fresh herbs, including parsley, chlorophyll degradation was shown to be retarded by an atmosphere containing 5% carbon dioxide and accelerated by ethylene relative to an air packed control, (Aharoni *et al.* 1989). Similar results were shown by Apeland (1971) who identified a close relationship between colour retention, using subjective colour assessment, and low respiration rates (measured using CO_2 production). Also noted in this study were the unfavourable effects of leaf wash treatment. In a more systematic study on ethylene-enhanced senescence in spinach and

parsley, Philosoph-Hadas *et al.* (1989), recorded a similar increase in loss of chlorophyll with exogenous ethylene and showed the inhibitory effect of silver ions and aminooxy acetic acid. Interestingly they also showed a sharp periodic increase in endogenous ethylene shortly after leaf detachment along with the well-documented climacteric rise at longer storage times. It was concluded that the burst of wound ethylene may promote senescence and therefore it might be anticipated that tissue handling procedures which parallel the mechanical stress of leaf detachment may adversely effect quality. Finally, in irradiation studies on parsley involving chlorophyll monitoring, little or no effect from the stress was observed (Josimovic 1983).

(ii) **Carotenoids**

Chemically, the carotenes are C_{40} hydrocarbons containing a conjugated sequence of carbon double bonds responsible for the typical spectral shift and their characteristic yellow-red coloration. Xanthophylls, descriptively included within the carotenoid group, result from the insertion of an oxygen function into the carotene molecule, with the effective maintenance of the conjugated moiety. Carotenoids are located in the chloroplasts and chromoplasts of higher plants and function as light harvesting/protectant molecules in close association with chlorophyll and the mediation of pollination/seed dispersal respectively. The chloroplast contains four specific carotenoids, namely lutein (40-57%), β-carotene (25-40%), violoxanthin (9-20%) and neoxanthin (5-15%; Gross 1991). In parsley, these chloroplast carotenoids dominate (Francis *et al.* 1989), with lutein reported at 10.2mg/100g fresh wt and representing one of the highest levels in vegetables. Information on the effect of processing on carotenoid levels in parsley is limited. However, in studying chlorophyll catabolism in senescing parsley leaves, Amir-Sapira *et al.* (1987) recorded a series of HPLC chromatograms including the carotenoids and showing a selective decay over a 6 day period at 25°C in the dark. Unfortunately only partial identification of the components was made and no quantitation of the data was offered. In a second study using fresh parsley, Nutting *et al.* (1970) showed an increase in both β-carotene and xanthophylls after steam blanching at 100°C for 25 seconds, and offered a number of explanations for the unexpected data. Also, in investigating the effect of blast freezing at -32°C, they showed a decrease in the β-carotene and xanthophyll levels compared to unfrozen material, for the unblanched sample and no significant change

for blanched samples.

1.5.3 Taste Chemicals (Taste)

Specific information on taste components in parsley is very limited. In recent years, however, considerable understanding on the molecular recognition of sweet and bitter compounds has emerged, which would allow the prediction of taste characteristics from known endogenous chemicals (van der Heijden 1993). Although no specific components in parsley have been directly related to bitterness/taste, with the exception perhaps of the flavonoid, dihydrochalcone, there are many chemical groups present in parsley which contain examples of bitter compounds (eg aromatic hydroxy compounds, flavonoids, pyrazines and the terpenoids; Maga 1990, Chialva *et al.* 1990). So well represented is parsley in the occurrence of phenyl propenoid derivatives, (represented by the former two groups), that it has played a significant role in understanding this biochemical pathway (Hahlbrook *et al.* 1989).

In summary, although information on the important taste chemicals in parsley is not available it should be recalled that the quality/sensory data discussed in the previous section suggested that sharpness/bitterness was not a storage issue in frozen parsley and as such is not an immediate focus for this study.

1.6 Basis of Chemical/Biochemical Change in Parsley During Processing and Storage

The objective of this section is to highlight current understanding on the chemistry and biochemistry relevant to the dynamics of key quality chemicals in parsley. It also serves to identify secondary species associated with key quality chemical changes which can be considered as potential target analytes when selecting and developing analytical methods, see Chapter 3.

In the preceding section, the flavour chemical composition of parsley has been identified and the effect of various forms of processing stress on selected chemicals has been described. In this section, the aim is to extend this picture by considering each chemical or group of chemicals to establish the likely routes of formation and loss such that they

can be assessed in terms of their relevance to the quality change within the tissue during processing and in terms of their relevance to the enzymic bias of the study. For volatiles, a detailed consideration of all 105 components identified, would be limiting and unnecessary at this point. Instead five 'key' chemical groups, relevant to parsley, have been identified at this point for consideration:

Group I	Components which change during technological processing.
Group II	Key odour potent/quality potent components.
Group III	Components which represent a particular chemical class/route of formation and are of significance due to their odour threshold/gravimetric standing.
Group IV	Secondary species associated to key quality chemicals change.
Group V	Enzymes associated to key quality chemicals change.

The chemistries and biochemistries of these chemicals (Table 1.4) will be reviewed in the context of routes of formation and degradation, in the following section.

1.6.1 Volatiles (Aroma)

Three primary routes of volatile formation and loss have been identified as:-

(i) endogenous enzymes in the formation of secondary products in healthy pre-harvest tissue.

(ii) post harvest stress induced endogenous enzymes, and

(iii) chemical routes.

Each aroma chemical will be subject to one or more of these processes, the balance of which will dictate the gravimetric status with time and thus its contribution to the quality. Additional loss may occur by physico-chemical partitioning between the phases and is likely to have a significant role. Also evaporative loss and partitioning into packaging materials will need to be considered in the overall scheme of processing to achieve improved quality.

(i) Secondary Product Formation

The secondary products group is effectively responsible for the 'base' flavour of the tissue prior to any processing stress, and is therefore a natural target for analysis.

Table 1.4 Important Chemicals Related to the Quality of Frozen Parsley

Chemical Category	Quality Category	Chemical	Relationship to Quality
Group I Key Quality Chemicals Effected by Processing	Volatile	(menthatriene)	terpeny aroma
	Colour	chlorophyll	fresh green colour
	Taste	?	
Group II Key Quality Chemicals	Volatile	AEDA chemicals see Table 1.2	see Table 1.2
	Colour	chlorophyll	fresh green colour
	Taste	?	
Group III Secondary Quality Chemicals	Volatile	apiol (E,Z)-1,3,5-undecatriene α-phellandrene sabinene cis 3-hexen-1-ol trans 2-hexenal p-cymenene methyl acetophenone p-cymene dimethyl sulphide	parsley-like, herbaceous aroma fruity, balsamic aroma citrus, peppery aroma oily, woodl aroma green aroma green, fruity aroma terpeny, hay-like aroma pungent floral aroma citrus aroma rotten cabbage aroma
	Colour	pheophytin chlorophyllide	olive green colour fresh green colour
	Taste	trihydroxy octadecanoic acid	bitter taste
Group IV Chemicals Associated to Quality Chemical Change		apigenin umbelliferone coumaric acid dihydro fumaric acid ascorbic acid tocopherol neoxanthin violoxanthin lutein β-carotene galacto-lipids phosho-lipids unsaturated FFA	(see Section 1.6.4) co-oxidant-POD co-oxidant-POD co-oxidant-POD active oxygen species source antioxidant antioxidant, quencher quencher quencher quencher precursor, antioxidant, quencher precursor for volatiles precursor for volatiles precursor, co-oxidant-LOX
Group V Enzymes Associated to Quality Chemical Change		peroxidase (POD) lipoxygenase (LOX) polyphenol oxidase (PPO)	see Section 1.6.5

It is dependent on the rate of biosynthesis, accumulation and volatile catabolism as is known to occur for terpenes and represents the dynamic nature of flavour in living tissue, (Loomis *et al.* 1980). The relevance of this group, to a study focusing on post harvest treatment, is their contribution to quality change during processing and storage with regard to any change in the balance of these processes as respiration alters (Apeland 1971). Certainly *de novo* synthesis is well established in the post harvest ripening of fruit (Tressl *et al.* 1986) and has also been suggested for parsley root (Kraxner *et al.* 1980). An understanding of the biochemistry of these pathways (substrate, enzyme, products) and their dynamics would be of significance in trying to establish the potential for flavour chemical change after harvest. For example, volatiles are often maintained and transported in a non-volatile form in-tissue, eg. pyrophosphates and glycosides, which can be subsequently cleaved to release the volatile moiety (Stahl-Biskup 1987). Establishing the concentration of such forms and the effect of processing on them is a major issue, most widely researched within the wine industry (Williams *et al.* 1992). If we consider the five groups of chemicals defined earlier (Table 1.2, Table 1.4) then, 15 compounds are likely to be products of secondary metabolism and thus part of the tissue 'base' flavour as defined earlier. These components are produced enzymatically via four different pathways, namely terpenoid and phenyl propanoid synthesis, and the metabolism of amino acids and lipids (β-oxidation).

Considerable work has been directed to terpenoid synthesis and there are a number of comprehensive reviews (Croteau 1985). Of particular relevance to this study is the unified stereochemical model proposed by Croteau (1986) based on the enzymology of monoterpenes and model studies from various sources. The above model details the pathway of formation of the acyclic monoterpene, myrcene and the cyclic monoterpenes sabinene and limonene. Although this model does not present sabinene as the pyrophosphate, other studies working with marjoram have identified this form (Fischer *et al.* 1988). The enzymes responsible for the cyclization of the universal allylic precursor, geranyl pyrophosphate (Croteau 1986) are the cyclases or more correctly termed isomerase-cyclases, as the isomerisation is a key preliminary stage. To date some 50 cyclases have been identified and it is assumed that all operate by a similar general mechanism although each individual cyclase is capable of producing a simple derivative or a positional isomer of the same skeletal type and may apply in our study to terpinolene

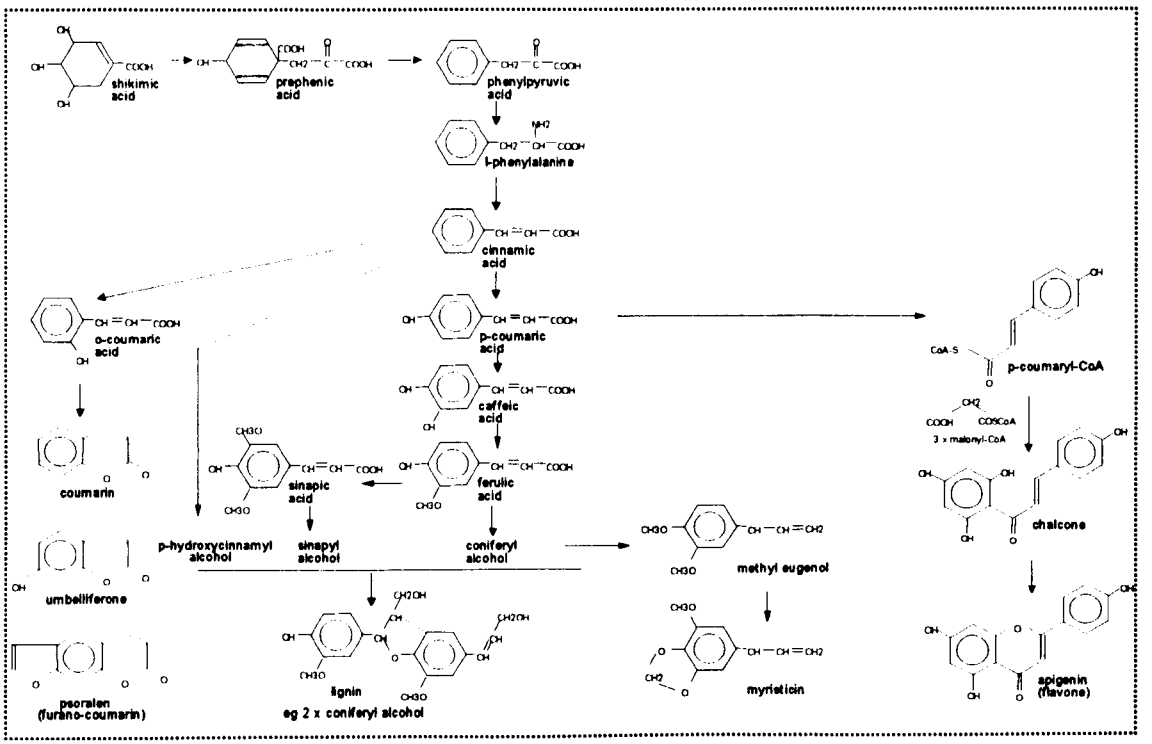
and α/β-phellandrene. It is worth noting that conversion between positional isomers of this type can also occur chemically and this is addressed later. γ-Terpinene, cited in the above model, has been shown to undergo further enzymic conversion via a dehydrogenase to give p-cymene (Poulose *et al.* 1978). The relevance of such an enzymic change to this study is (a) the introduction of a carbon-carbon double bond into the menthene skeleton, a process which must occur in the formation of the important conjugated but non-aromatized triene, menthatriene, and (b) the potential analogy to the aromatization of menthatriene to p-cymenene. Interestingly, in novel studies on the volatile constituents of callus, cell suspension and differentiated plant tissue of *Petroselinum Crispum*, Gbolade *et al.* (1989) showed menthatriene and p-cymenene to be present at the callus stage. Significantly, microscopical examination of these cultures established the absence of glandular or secretory structures. Analysis of the growing medium showed the absence of volatiles and seemed to suggest that these types of secondary products were produced in non-specific cells in a form which was retained by an active mechanism (Stahl-Biskup 1987). Interestingly and in contrast to this, the mentha species, *mentha spicata*, biosynthesizes and accumulates the principal monoterpene, carvone, in glandular trichomes extending from the epidermis of the leaf (Gershenzon 1989). The relevance of this information relates to the location and availability of aroma chemicals and thus the potential site of reaction with respect to degradation and change. In healthy tissue the essential oil, although produced in non-specific cells and perhaps transported and accumulated in the oil ducts referred to in the earlier histology section, appears to be actively retained as no aroma is detected if intact plants or sprigs are assessed sensorially. If, however after harvesting, the plant's respiration changes, preventing the maintenance of this active process, then the chemicals will distribute based on chemical energetics and may enter new reaction environments. In addition to this biochemically initiated redistribution, physical processes may also be pertinent as the cell is frozen and ice crystals rupture cells and decompartmentalize species. Certainly in qualitative laboratory tests, fresh whole parsley sprigs of low aroma intensity, when immersed in liquid nitrogen and subsequently allowed to thaw, yield a very strong characteristic parsley aroma.

The two oxygenated acyclic monoterpenes, which conclude this section, citronellol and linalool are formed enzymically via a NADPH dependent enzyme from geraniol and directly from geranyl-pyrophosphate respectively, although the enzyme system in the latter conversion has not been demonstrated and may operate in parallel to a non enzymic

conversion reported by George-Nascimento *et al.* (1971).

The second biosynthetic route relevant to parsley is the shikimic acid/phenyl propanoid pathway. This pathway, summarized in Figure 1.7, is responsible for a series of aromatic chemical categories including coumarins, chalcones, flavonoids, soluble esters and lignins (Herbert 1989; Robinson 1963), as well as a number of volatile products relevant to this section.

Figure 1.7 Biosynthesis of Phenyl Propanoids



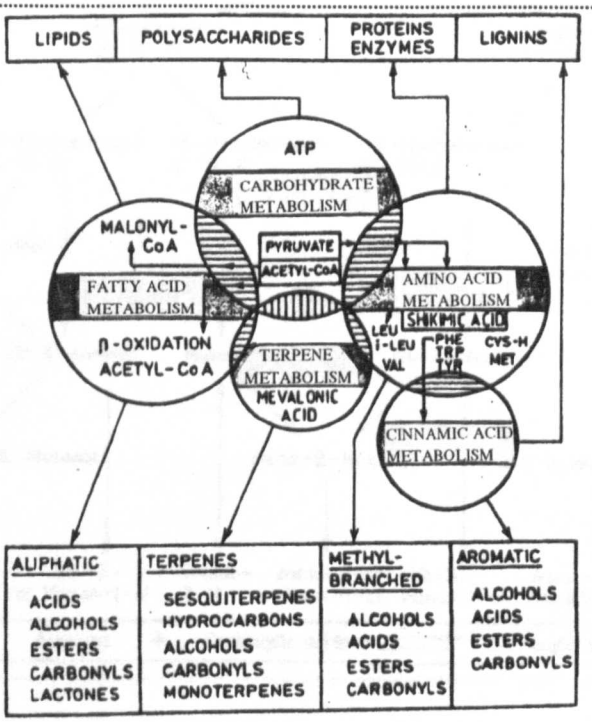
Parsley has played a significant role in the understanding of this pathway (Hahlbrock *et al.* 1989) although the focus has been towards the non-volatile components. In labelling experiments, banana slices incubated with (1 - ^{14}C) caffeic acid showed methyl eugenol as a product (Tressl *et al.* 1975). In a separate study involving *Perilla frutescens*, methyl eugenol was shown to be a common precursor of elemicin and myristicin, the latter material being a precursor of dill apiol (Nishizawa *et al.* 1988).

The third biosynthetic category of relevance to parsley, is amino acid metabolism which forms the precursors of methyl substituted aroma compounds (Tressl *et al.* 1986).

Although the literature contains no direct reference to the group II component, methyl 2-methyl butanoate, studies have identified the '3 isomer' along with other similarly branched butanoates in labelling experiments utilizing (U-^{14}C)L-leucine and banana slices. Amino acids have been also proposed as the source of 2-methoxy pyrazines in plants via a biosynthetic amidation/condensation/methylation sequence (Murray *et al.* 1970; Gallois *et al.* 1988; Leete *et al.* 1992), although again no information on the '3 isomer' is available. The final biosynthetic pathway for consideration is β-oxidation of fatty acid involving the sequential cleavage of C2 units and responsible for a range of volatile aliphatic alcohols, esters, acids and hydrocarbons. Tressl *et al.* (1986) proposed such a sequence for the biosynthesis of (E,Z)-1,3,5-undecatriene, a group III component.

In the above discussions, the focus has been towards secondary product, aroma volatiles relevant to parsley. In a more general context, Tressl *et al.* (1986) summarized secondary product pathways showing the interplay between aroma volatiles and primary metabolism in an excellent and much referenced scheme (Figure 1.1, Figure 1.8).

Figure 1.8 Biogeneration of Fruit Volatiles (Tressl *et al.* 1986)



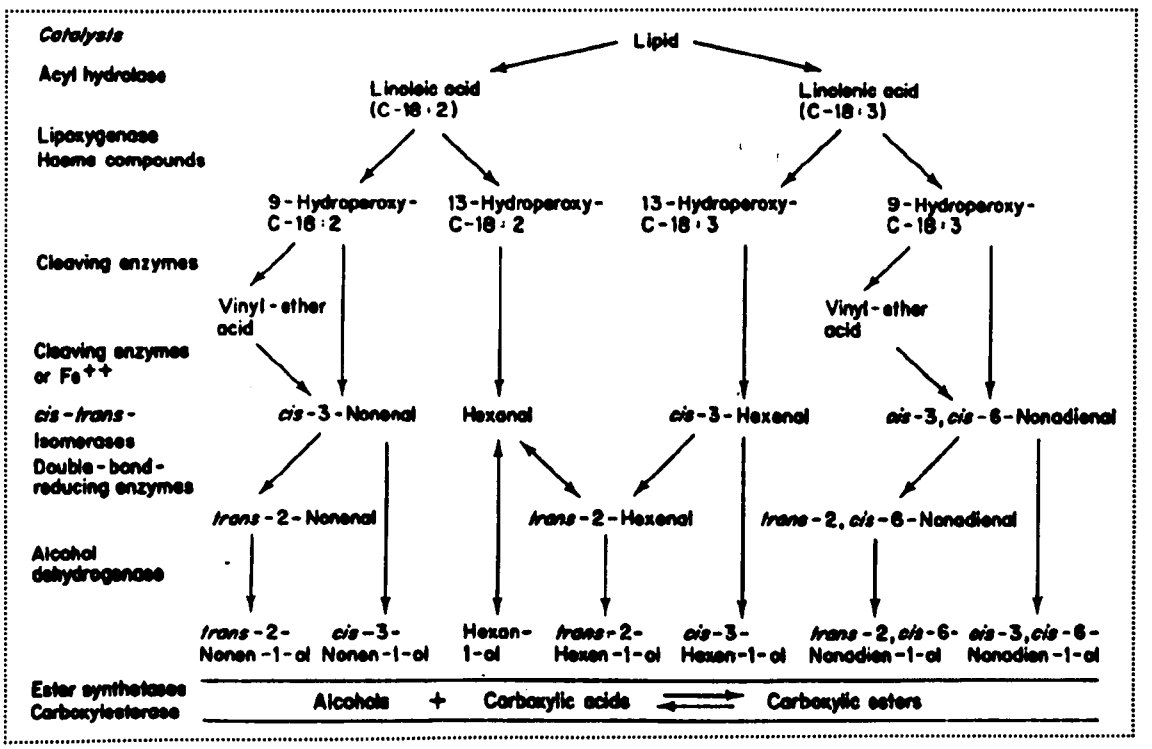
The focus in this section has been to consider biogeneration of the target species. However

it is equally relevant to consider how these react further via interconversions and catabolic pathways. For example, beta oxidation of undecatriene, formation of hexenyl acetate from hexenol via a transesterase and the enzymic conversion of p-cymene to thymol, all of which have been reported in parsley (Appendix 1.3).

(ii) Stress Induced Enzymic Processes

The second route by which parsley volatiles may be generated is via stress related endogenous enzymes. Of primary importance to flavour quality is the lipoxygenase pathway, triggered by maceration or cell damage (Gardner 1988). Lipoxygenase products are generally deleterious, however, in certain tissues namely tomatoes and some fruits, they are beneficial provided the levels are not excessive (Whitaker 1991). Of interest to this section is the formation of the important sensory components, cis 3-hexen-1-ol and trans 2-hexenal, (group III components, Table 1.4).

Figure 1.9 Formation of C6 and C9 Volatiles from Plant Lipids (Eriksson 1979)



Lipoxygenase initially operates on linolenic acid to yield the 13-hydroperoxide, hydroperoxide lyase then selectively cleaves the molecule to produce cis 3-hexenal which

following the action of alcohol dehydrogenase is converted into cis-3-hexen-1-ol. Alternatively cis-3-hexenal may be converted into the trans-2 isomer via the isomerase (Eriksson 1987; Figure 1.9).

The support for lipoxygenase being entirely responsible for the C6 aldehydes and alcohols in parsley is still open to debate even though many of the key elements are established. Baardseth *et al.* (1987) reported the presence of lipoxygenase in parsley albeit at the lowest level of the 22 vegetables investigated, whilst Kim *et al.* (1990), MacLeod *et al.* (1985), reported the volatile products of the pathway. The subject of off flavour generation and the role of enzymic degradation of polar lipids in frozen parsley has been extensively studied by Duden (1984,1985) and coworkers (Scholz 1982; Fricker 1981; Hubner 1981), and provides a firm basis from which to plan experimental work for this thesis. The philosophy adopted in their study was to hypothesise that, if polar lipids are the source of flavour change on frozen storage, it should be possible to map the decay of substrates, and obtain a stoichiometric relationship with the products for all the various enzymes involved in a degradation cascade. In their study, they considered the two main classes of lipids in leaf material, galactolipids and phospholipids (Hitchcock 1971), and monitored the loss of the parent molecule along with two key intermediates of the lipoxygenase cascade, free fatty acids and hydroperoxydienoic acids (HPDA; Eriksson 1979; Figure 1.9).

Duden *et al.* (1981, 1982) showed extensive loss of the parent galactolipids, monogalactosyl diglyceride (MGDG, 100% after 1 month) and digalactosyl diglyceride (DGDG, 75% after 2 months), and the phospholipid, lecithin, (80% after 5 days), when stored at -12°C However no stoichiometric relationship was observed with either free fatty acid or HPDA formation. Based on the lack of a stoichiometric relationship and a knowledge of the starting material, phosphatidic acid (PA) and 6-acyl MGDG were monitored as alternative intermediates of phospholipid and galactolipid breakdown. From these data, a stoichiometric relationship was obtained for phospholipids and it was concluded that phospholipids were degraded by the action of phospholipase D to phosphatidic acid and the corresponding base. However free fatty acids are not released and therefore not a source for the lipoxygenase cascade under the conditions investigated. For galactolipids, the 6-acyl MGDG showed a transient response, suggesting that, although it may be the first stage in breakdown, subsequent pathways must exist.

In concluding their study, Duden (1984) states that enzymic processes play a major role in the development of off flavour in unblanched frozen parsley and that non enzymic processes proceed more slowly and are completely masked by the former. From the earlier chemical studies, Duden concluded that the hydrolytic-lipoxidation pathway had not been verified, based on the lack of a link between lipid loss and free fatty acids formation and the very low levels of HPDA, and assuming that lipoxygenase does not directly attack unhydrolysed lipids (Galliard *et al.* 1975; Brockmann *et al.* 1977). However from the sensory work, they postulate that the lipoxygenase cascade may be responsible for the 'grassy' character based on there being sufficient levels of HPDA to generate the very odoriferous C6 volatiles. With respect to the 'hay like' character, the assumption is made that enzymic processes involving anaerobiosis and fermentative change are responsible although specific processes were unknown to the authors. It is tempting at this point to offer some consideration to the enzymology responsible for this grassy to hay like transition. However it must be remembered that the data available, although of great significance, is based on sensory analysis only and complementary volatile analysis would be of great value. Nevertheless it is worth noting that the lipoxygenase enzyme can operate under an anaerobic environment to produce very different products to the normal aerobic mode, for example ketones, saturated and unsaturated hydrocarbons, (de Groot *et al.* 1975; Gardner 1988). It can be seen that under aerobic conditions HPDA's are generated which subsequently further cascade down to C6 aldehydes (grassy) whilst under anaerobic conditions lipid radicals are released from the enzyme and are involved in various rearrangement and further oxidation reactions to yield the volatiles cited above. In this context, oxygen content may be important and, although product is stored under aerobic conditions, a situation may arise whereby a tissue which has endogenous oxidative processes operating, for example the oxidoreductase enzymes, consumes oxygen locally to levels where anaerobiosis might occur.

In the preceding two sections on secondary metabolism and stress related enzyme systems, we have almost exclusively dealt with specific enzymes which are directly associated with a specific substrate-to-product conversion. However in plant tissues, many other enzyme systems are operative, which are also relevant to flavour quality, through a less direct or secondary role, for example peroxidases, ascorbic acid oxidase and superoxide dismutase. These will be discussed with regard to specific chemicals in subsequent sections of this

review, as was the format here, or in association with data generated within this programme (see Section 1.6.2).

(iii) Chemical Processes

The final route identified in the formation and loss of parsley volatiles is via chemical processes. In view of the reactive nature of many of the endogenous flavours and flavour precursors, and the changes in environment resulting from technological processing, these processes are likely to have a major effect on flavour quality.

Only a limited amount of information is available on the chemical reactions involving volatiles in parsley itself. However, considerably more information is available from other plant tissues and this information can be applied to the chemistry of formation and subsequent reactions of the components present in parsley. The main chemical reactions relevant to parsley under typical processing regimes are:

- oxidation involving factors like metal ions, haem compounds, free radicals (ie autoxidation) and singlet oxygen (ie photoxidation) .
- dimerization/polymerization
- acid catalysed reactions including hydrolysis, hydroxylation, isomerization and rearrangements.

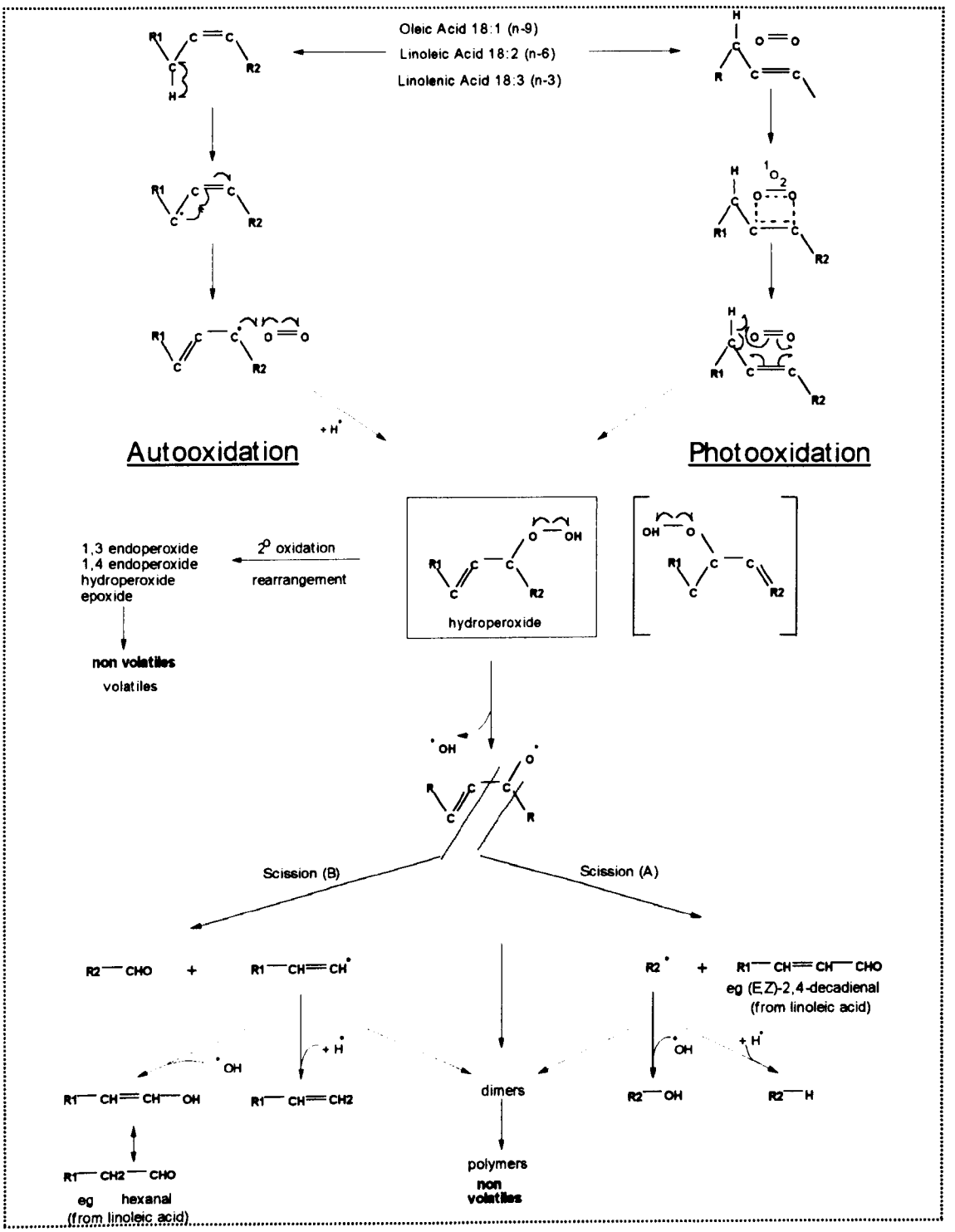
These manifest themselves, as do biochemical processes, either in (a) the formation of volatiles from non-volatile precursors, (b) conversion to another volatile (with different organoleptic character) and (c) formation of non-volatile products.

Most of the oxidative chemistry of parsley, as with many plant tissues, results from the presence of active oxygen species (Foote 1985) and the carbon-carbon double bond where, the two main oxidative mechanisms applicable to unsaturated lipids are free radical chain reactions and singlet oxygen addition. In both these cases hydroperoxides, and/or in the event of a conjugated or polyunsaturated systems endoperoxides, are formed which undergo subsequent fission to form hydrocarbons, epoxides, ketones, alcohols, aldehydes, acids and even furans, (Figure 1.10; Grosch 1987; Frankel 1982; Min 1988).

The type of compound formed depends on the initial reactant, the oxidative route to hydroperoxide formation and the environment under which fission occurs (ie pH, metal ions, temperature, inhibitors; Eriksson 1987). If we consider group II chemicals, ie those obtained from AEDA analysis, Table 1.2, we see that oxidative chemistry is likely to play

a significant role, as 5 of the 13 components probably have their origins in the chemical oxidation of unsaturated lipids/carotenoids. As stated earlier, the reaction products of lipid oxidation are clearly a function of the unsaturated fatty acids present.

Figure 1.10 Autoxidation and Photooxidation of Unsaturated Lipids



In parsley the following fatty acids have been reported (Balbaa 1975; Shaath *et al.*1988) :-

cis-9-tetradecenoic acid	14:1 (n-3)	myristoleic acid
cis-9-hexadecenoic acid	16:1 (n-7)	palmitoleic acid
cis-6-octadecenoic acid	18:1 (n-12)	petroselinic acid
cis-9-octadecenoic acid	18:1 (n-9)	oleic acid
cis-9,12-octadecadienoic acid	18:2 (n-6)	linoleic acid
cis-9,12,15-octadecatrienoic acid	18:3 (n-3)	linolenic acid

(E,E)-2,4-decadienal has been shown to originate from linoleic acid. The mechanism involves abstraction of hydrogen at C_{11} and delocalization of the radical over the five carbon diene moiety, allowing isomerization of the carbon double bonds. Addition of oxygen to the C_9 carbon produces the corresponding 9-hydroperoxide which subsequently breaks down by scission of the C_8-C_9 bond to yield 2,4 decadienal (Figure 1.10; Frankel 1985). 1-Octen-3-one and (Z)-1,5-octadien-3-one are likely to originate from linoleic acid and linolenic acid respectively, (Bading 1970; Grosch 1987). (E)-6-decenal however is not reported although it seems likely that it is formed from some sort of lipid oxidation pathway.

In an earlier section, the enzymic generation of stress related volatiles eg the group III components cis-3-hexen-1-ol and trans-2-hexenal was considered. Clearly a considerable degree of commonality exists between an enzymic aldehyde/alcohol generation cascade and a chemical one, to the point where both processes can run in parallel and perhaps even interchange, with the intermediates from one feeding the other and *vice versa.* Another example of a common product is (E,E)-2,4-decadienal as cited in the chemistry above and reported in enzymic studies involving soybean lipoxygenase and linoleic acid (Fischer *et al.* 1977).

Another class of unsaturated lipid material of direct relevance to the odoriferous components in parsley is the carotenoids and, as with other unsaturated material, biochemical (Grosch *et al.* 1976; Enzell 1985) and chemical oxidation apply (Drawert *et al.* 1981; Simpson 1985), although little is known of the biochemistry of carotenoid catabolism (Parry *et al.* 1991). Of particular interest is the degradation of β-carotene to β-ionone. Although β-carotene is not a direct substrate for the lipoxygenase enzyme there

appears to be a co-oxidation sequence in which a biochemically generated peroxy radical acts as the initiator in a classical autoxidation sequence (Grosch 1976; Stevens 1970), as supported by the effective action of antioxidants (Goldman *et al.* 1983). Ouyang *et al.* (1980) suggests an alternative mechanism where an oxidative pathway initially involving singlet oxygen addition via the 'ene' reaction is followed by homolytic fission to produce β-ionone. In this reaction, singlet oxygen ($^1O_2$) is chemically quenched by β-carotene however this quenching process competes with the favoured physical quenching mode of carotenoid (ie $^1O_2$ + β-carotene → $^3O_2$ + ^{3}β-carotene + heat) and is likely to occur only after prolonged irradiation (Min *et al.* 1988).

It is interesting to note that water activity (Aw) is important in influencing the oxidation rate of carotene, as in general lipid oxidation, having a beneficial effect at intermediate Aw and a detrimental effect at low Aw (Rockland *et al.* 1987; von Elbe 1987; Leung 1987). Of particular interest to this study is the consequence of frozen storage with respect to Aw and the rate of oxidative change and whether the recent theories on glassy states are more applicable (Levine *et al.* 1988; Slade *et al.* (1991).

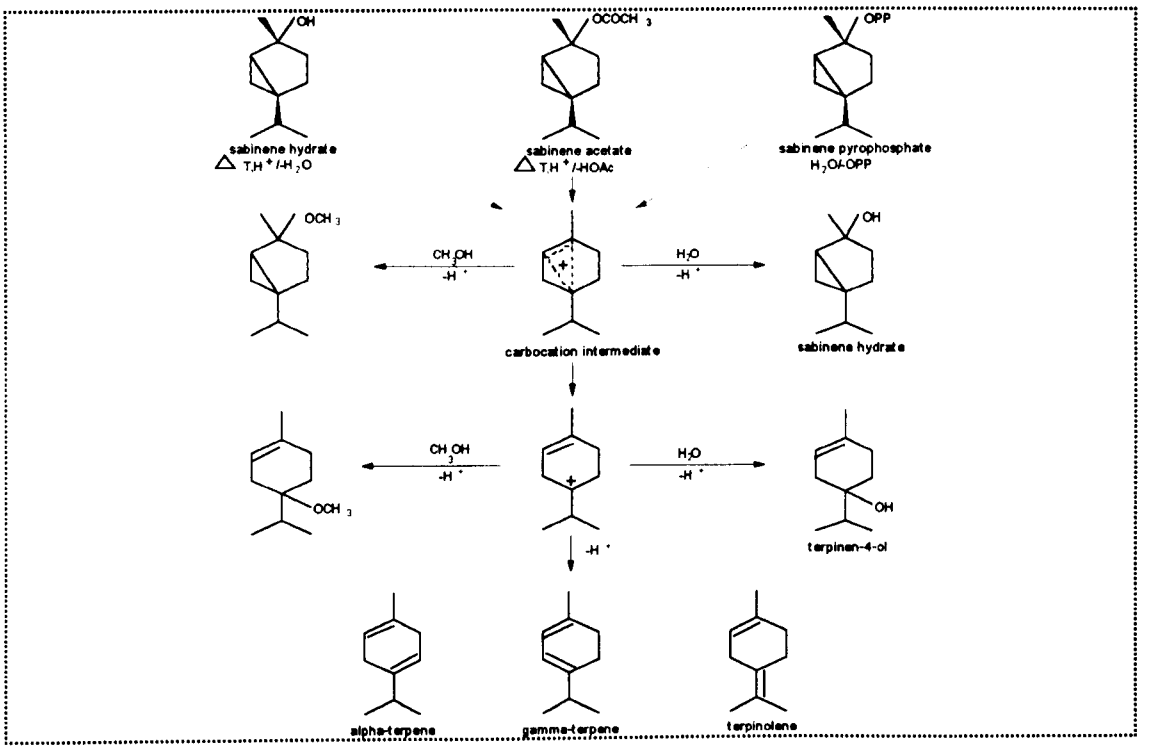
Related to the carotenoids discussed above, are the mono- and sesqui-terpenes. These groups of volatile compounds are lipophilic in nature and contain isolated and conjugated carbon-carbon double bonds and are susceptible to oxidative attack. Menthatriene, a group I and II component, has been cited to undergo oxidative change/loss during the frozen storage of parsley with the formation of the 1,4 endoperoxide and 1,2-3,4 diepoxide derivative (Nitz *et al.* 1980; Appendix 1.3). Structure elucidation and reaction mechanisms were determined in model studies involving sensitized photoxidation of menthatriene where 1,4 cyclic addition of singlet oxygen to the 1,3 conjugated diene ring occurred. Further irradiation and/or thermal treatment lead to the reported diepoxide and ketoepoxide (Nitz *et al.* 1989a). From these studies it is interesting to note the apparent absence of hydroperoxides as minor derivatives, produced by the competing addition of singlet oxygen ('ene' reaction) across single carbon double bonds (eg. C1, C2 position), as reported for similar conjugated monoterpenes (eg. α-phellandrene and ∝-terpinene). However this may be due to their subsequent conversion to the aromatized form of menthatriene namely, p-cymenene (von Matusch *et al.* 1989,1990). p-Cymenene (4-isopropenyl-1-methyl benzene) has been reported in relatively high levels in parsley leaf

(Kim *et al.* 1990; Table 1.3; Group III compound), as well as being present in the model photoxidation studies by Nitz *et al.* (1989a). As an alternative mechanism to hydroperoxide degradation, as discussed above, Spraul *et al.* (1991) postulated a radical sequence involving the sequential abstraction of hydrogen from menthatriene by oxygen with the formation of a hydroperoxy radical and hydrogen peroxide respectively. Although singlet oxygen generated by photochemical means has been shown as an *in-vitro* mechanism relevant to the conversion of menthatriene to the endoperoxide, it remains unclear as to its role *in vivo* (Nitz *et al.* 1989b). Certainly a number of endogenous photosensitizers are well established and relevant to parsley ie chlorophyll, pheophytins, (Min *et al.* 1988), as well as the furanocoumarins (Knox *et al.* 1985; Ceska *et al.* 1987), however their relevance to parsley tissue stored in the dark at frozen temperatures is uncertain. Interestingly a similar study on wormseed fruit, considering the formation of the 1,4 endoperoxide, from α-terpinene (Appendix 1.3), an iodide-peroxidase catalyzed biosynthesis was shown to operate to the exclusion of a singlet oxygen or superoxide mechanism (Johnson *et al.* 1984). On a practical note, it is worth recording that many of these endoperoxides and epoxides are thermally labile and sensitive to polar phase GLC columns where 100% degradation has been observed (Nitz *et al.* 1989a).

Another class of chemical reactions, likely to be significant to parsley are acid catalysed reactions. Reaction of this type fit into three categories, hydrolysis, hydroxylation and rearrangements/isomerizations and are very sensitive to the preparation procedures involved in flavour analysis. Acid catalyzed hydrolysis is responsible for the liberation of volatiles from their bound form by cleavage of the ester linkage in phosphate esters, eg sabinene pyrophosphate, and the glycosidic bond in glycosides, eg linalool glycoside. In the case of the glycoside, the corresponding alcohol, linalool, is formed. However because of the nature of the pyrophosphate group and its characteristic to act as a good leaving group, either the hydrocarbon or the alcohol may result. These processes may also be achieved enzymically. The potential contribution of these processes to the composition of a flavour cannot be underestimated as clearly demonstrated by the studies of Fischer *et al.* (1988). In their work on marjoram they showed that only three primary components exist in intact tissue, (sabinene, sabinene hydrate and sabinene pyrophosphate), however an extensive array of typical monoterpenes was formed during aqueous distillation, partly due to hydrolysis of the bound pyrophosphate and partly due to rearrangement of the

liberated/free flavour forms. These rearrangements arise from the protonation of the tertiary alcohol to yield the oxonium ion which splits to form a stabilized carbocation. Subsequent isomerization, rearrangement and hydration reactions readily occur via charge delocalization followed by either deprotonation or hydroxylation and lead to the complex picture summarized in Figure 1.11.

Figure 1.11 Proposed Reaction Scheme for the Formation of Monoterpenes in Sweet Marjoram (Fischer *et al.* 1988)



Many of these products and certain of the starting materials are observed in parsley. Consideration will need to be given to establish which are artifacts of analysis, as concluded for marjoram, and which are present in intact tissue or formed during technological processing. The final type of chemical reaction for consideration is dimerization. In the case of menthatriene and myrcene the presence of a 1,3 diene and a dienophile component allows a reversible Diels-Alder reaction to occur. Various products of this reaction have been observed in parsley oils (Shaath *et al.* 1988) but their status and relevance in tissue is unclear. Another class of volatile components of general importance in vegetables are those containing sulphur. Dimethyl sulphide (group III component) is the only sulphur compound reported to date in parsley (Freeman *et al.* 1975) and may be formed chemically or enzymically from the amino acid derivative, methylmethionine

sulphonium salt (Schwimmer *et al.* 1972; Golubev *et al.* 1987).

1.6.2 Pigments (Colour)

The main pigment classes within vegetables and fruits are the green chlorophylls, the yellow to red carotenoids and the red to violet anthocyanins. During the vegetative/climacteric period these pigments are in a state of flux where the visually dominant chlorophylls are degraded unmasking the underlying carotenoid pigments which themselves may enter a *de novo* synthesis stage (Gross 1991).

(i) Chlorophylls

Chlorophyll degradation can be viewed from a number of perspectives in order to establish an understanding relevant to a food quality study of this type. Three areas of investigation can be identified, (i) chlorophyll turnover in healthy growing tissue, (ii) changes during the relatively orderly process of leaf senescence and (iii) changes present in dead/non respiring tissue after physical stress (Halliwell 1984). In this section, a brief overview of the status of chlorophyll loss in vegetables/plants as a whole is undertaken followed by a summary of the information specific to parsley.

In healthy plant tissue, chlorophyll content reflects the dynamics of the anabolic and catabolic processes and gives rise to a certain turnover involving daytime synthesis and nighttime degradation. This turnover has been shown to vary from several hours to several weeks depending on the species and maturity of tissue (Hendry *et al.* 1987). Although the biosynthesis of chlorophyll is well established (Castelfranco *et al.* 1983), the mechanism and products of catabolism and any reutilization is largely unknown (Hendry *et al.* 1987). Plant leaf senescence and the associated loss of chlorophyll and the unmasking of the red/orange carotenoid/flavonoid pigments typical of autumn foliage is one of the most visually obvious biochemical processes. Although many investigations have been conducted on this subject, significantly no stoichiometric relationship between chlorophyll loss and degradation products has been made (Hendry *et al.* 1987). In citrus fruit, chlorophyll loss has been shown to correlate with the dephytylated species, chlorophyllide, present at low levels, and shown to be associated with chlorophyllase

activity (Amir-Shapira *et al.* 1987). Interestingly the author also showed pheophytin rather than chorophyllide accumulation in parsley leaves and suggested an alternative mechanism was operative. The chlorophyllase enzyme is reported to co-exist with the chlorophyll-protein complex within the thylakoid membrane and is maintained in an inactive/stable configuration by adjacent lipids and conversely activated by Mg^{2+} levels (Lambers *et al.* 1985). Disruption of this membrane provides the necessary exposure of substrate for the reaction to proceed. In *in-vitro* studies, mechanical disruption or solubilization of isolated thylakoid membranes with detergents has shown complete hydrolysis of chlorophyll by chlorophyllase (Amir-Shapira *et al.* 1986). However chlorophyllase activity with the isolated chlorophyll-protein complex produces only 40% hydrolysis (Schoch *et al.* 1987). In studies *in-vivo*, only low levels of the hydrolysis product, chlorophyllide, are observed. This lack of accumulation has recently been attributed to it's reactive nature and subsequent degradation arising from oxidation and fission of the porphyrin ring (Peisker *et al.* 1989; Matile *et al.* 1989). In a comparison of parsley leaves at an early stage of senescence held subsequently under oxygen and anoxic conditions, typical low levels of chlorophyllide were recorded under oxygen. However in anoxic conditions, relatively high levels of both chlorophyllide and the dechelated form, pheophorbide, were obtained (Peisker *et al.* 1989) suggesting chlorophyllide may be a reactive intermediate degraded further by the action of oxygen. An extension to the mechanism of chlorophyllase action was the work performed following phytol location in senescing leaves (Peisker *et al.* 1989). In this study, little free phytol was observed; rather phytol was found to be esterified with linolenic acid suggesting a transesterase activity for chlorophyllase. In view of the low levels of chlorophyllides and their similar coloration to the parent molecule, it is unlikely that they directly feature in any observed colour change. As an alternative mechanism, it has been suggested that free radicals or superoxide anion, generated via either peroxidase or lipoxygenase activity are responsible for modification/bleaching of the chlorophyll within the protein complex (Lynch *et al.* 1984; Imamura *et al.* 1974). This molecular change in chlorophyll is thought to be related to a conformational change in the associated protein resulting in the activation of protease and subsequent proteolysis (Thompson *et al.* 1987). This hypothetical sequence offers an explanation for the highly correlated chlorophyll/protein degradation in senescing chloroplasts and, as bleaching is involved, would explain the unmasking of the carotenoids although little evidence for these degradation products is available. Additional support for the role of lipoxygenase

is the localization of lipolytic acyl hydrolases in the chloroplast and their role in the liberation of free fatty acid necessary for lipoxygenase action (Yamauchi *et al.* 1987). Further support for lipid peroxidation as an initial stage to chlorophyll loss and proteolysis is given in a recent study on detached parsley leaves using a new fluorescent technique to detect lipofuscin-like compounds whilst monitoring amino acid and chlorophyll dynamics (Meir *et al.* 1992). Model studies on chlorophyll have established the sensitivity of this material to free radical oxidation in the formation of 13^2 hydroxy chlorophyll 'a', described as allomerization (Hynninien 1981).

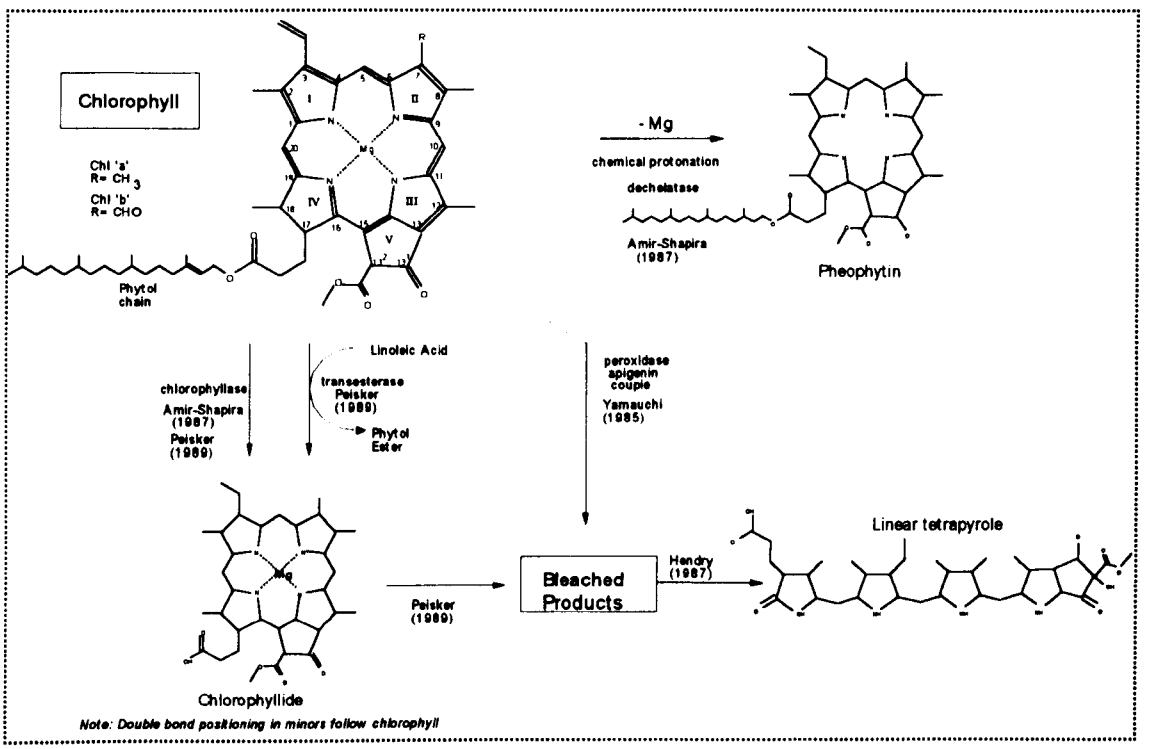
A third reported mechanism for the degradation of chlorophyll, involves the action of chlorophyll oxidase. This enzyme is activated by unsaturated fatty acids, and thus perhaps is linked to the lipase action cited above, however it is different to lipoxygenase in that it is not associated with linolenate hydroperoxide production nor does it uniquely require a 1,4 pentadiene moiety (Luthy *et al.* 1984). The reported product of chlorophyll oxidase is 13^2 hydroxy chlorophyll 'a' (Schoch *et al.* 1984), and represents a similar oxidation step to the free radical situation cited above. Another interesting feature in senescence studies is the role of α-tocopherol, which rises initially during the senescence period. α-Tocopherol is a natural free radical scavenger, present in chloroplasts, and is reported to play a role in the mediation of senescence through lipid and carotenoid stabilization. α-Tocopherol has been shown to increase in level during the senescence period either through biosynthetic utilization of phytol liberated in chlorophyll degradation (Rise *et al.*1989), or some other regulated pathway.

So far chlorophyll degradation in healthy tissue and during leaf senescence, has been considered; the third scenario of loss is within the dead/non respiring cell. Under this environment, many of the enzyme systems discussed earlier are likely to be relevant but many of the active defence mechanisms available in healthy and senescent material are not operative and will consequently affect the complex reaction profile/balance. Under typical food production regimes, healthy tissue is harvested and subjected to a unique combination of physical stresses. In many situations this leads to the production of non viable (dead) tissue for example, blanching, freezing and mechanical treatment. Under these conditions many additional chemical (and biochemical) processes are available. In mechanically disrupted/heat processed tissues, chlorophyll is protonated, resulting from the release of endogenous organic acids, with the removal of magnesium to produce

pheophytin, an olive green/brown pigment. Mild thermal treatment produces $C13^2$ epimers, whilst more aggressive heating causes loss of the carbomethoxy group to generate the recently identified pyropheophytin (Schwartz *et al.* 1983, 1990). Magnesium removal has also been reported to occur by biochemical means through a dechelatase (Houghen *et al.* 1982). Clearly, as for healthy and senescent tissue, lipid oxidation and its associated effects would be expected to be relevant to chlorophyll in dead/non respiring tissue.

In parsley, using intact tissue, mechanisms have been proposed to explain chlorophyll degradation, (Figure 1.12), and a fourth mechanism has been shown to exist in *in-vitro* experiments or under anoxic (no oxygen) conditions. In the introduction to this section the action of chlorophyllase, and the associated formation of the dephytylated species, chlorophyllide, was presented as the generally preferred mechanism for chlorophyll degradation.

Figure 1.12 Schematic Summary of Chlorophyll Degradation in Parsley



In parsley however, pheophytin and other transient phytylated derivatives are observed in *in-vivo* storage studies (Amir-Shapira *et al.* 1987). The mechanism for magnesium removal is unknown but it would be interesting to establish if a dechelatase was operative rather

than chemical protonation. The second *in-vivo* mechanism (and likely to be the most relevant to parsley) is the peroxidase bleaching scheme proposed by Yamauchi *et al.* (1980,1985). In the earlier study they showed chlorophyll and ascorbic acid loss to be negatively correlated with chlorophyllase and ascorbic acid oxidase activity, and positively correlated to peroxidase activity. They concluded that chlorophyll degradation depended on the action of peroxidase and that ascorbic acid was an inhibitor. In the later study, the pathway of the observed chlorophyll bleaching was shown to involve a peroxidase-apigenin oxidative couple, (Figure 1.12), similar to those reported involving lipoxygenase-linoleic acid (Orthoefer *et al.* 1973) and chlorophyll oxidase-unsaturated fatty acid (Schoch *et al.* 1984), however no degradation products were cited (Yamauchi *et al.* 1985). The reported oxidative mechanism for peroxidase (Robinson 1991) can be readily extended to include apigenin. This area will be the focus for further investigation within this thesis with the aim to investigate the role of peroxidase and to consider the issues associated with the flavonoid co-substrate, apigenin.

The final *in-vivo* mechanism cited for parsley stems from an interesting study following the level and location of phytol in senescent leaves. By analysing total phytol, free phytol and chlorophyll level, the levels of phytol esterified inside and outside chlorophyll have been calculated (Peisker *et al.* 1989). From the data, very little phytol is lost, rather phytol is transferred from chlorophyll to another species, perhaps by the transesterase activity of chlorophyllase involving linoleic acid as reported by Csuper (1971) for *Acer platanoides.* Interestingly, no dephytylated chlorophyllides were detected but, in the absence of oxygen, chlorophyllides were observed, as discussed earlier. In reviewing the behaviour of chlorophyll in parsley, it is worth stressing the reactive nature of the chlorophyll molecule to oxidative and photoxidative change which is an important consideration in the selection of analysis protocols. Significantly parsley has one of the highest levels of chlorophyll pigment of all vegetables, (total chlorophyll 198.2 mg/100g fresh wt; Gross 1991) and is matched by the proportionately high levels of the similarly located and stabilizing carotenoids, (15.8 mg/ 100g fresh wt).

In summary, it would appear that in certain aspects, parsley is atypical in terms of chlorophyll degradation and that a number of mechanisms are likely to apply depending on the history of the tissue and its environment resulting from the type of technological

processing applied. This situation fits well into the concept introduced at the beginning of the section, where turnover, senescence and changes within dead/non respiring tissue apply. Although the coupled peroxidase mechanism is felt to be the most relevant to frozen parsley, confirmation by establishing a stoichiometric relationship between bleaching products and reactants would be necessary. This area will be investigated further within this study.

(ii) Carotenoids

Carotenoid degradation has been discussed in the context of being a precursor to the key quality volatile, β-ionone. These oxidation mechanisms are relevant to the loss of carotenoids. However as these pigments are not dominant in green leaf tissue, they are unlikely to effect colour quality directly but, as carotenoids are protectants, they may have an indirect role in quality chemical retention and will be discussed in Section 1.6.4.

1.6.3 Taste Chemicals (Taste)

In the above sections, biochemical and chemical oxidation mechanisms have been cited as key degradative pathways. One effect of lipid oxidation on linoleic acid is the formation of the 9-hydroperoxide and 13-hydroperoxide, which have been shown to degrade further under a Fe(III)-cysteine system to 9,12,13-trihydroxy-10-octadecenoic acid (Gardner 1975), a known bitter principle.

1.6.4 Chemical Species Associated with Quality Chemical Change.

The discussions to date have highlighted a number of additional species which need to be considered in order to understand the important degradation processes and their inter-relationship in parsley. These species can be categorized into six groups, namely, precursors, co-oxidants, anti-oxidants, pro-oxidants, active oxygen species and photosensitizers.

Precursors

Unsaturated lipids esterified within phospho- and galactolipids are key precursors and play an important role in the generation of aroma potent chemicals as well as a source of free radicals capable of initiating further oxidation (eg. β-carotene → β-ionone), and in

mediating the action of chlorophyll oxidase.

Co-oxidants

The most relevant co-oxidants in parsley are likely to be the 'enolic' species which act as natural substrates for peroxidase, eg the flavonoid, apigenin. These, like the oxidized lipids, are potentially capable of initiating further oxidation within the tissue. Carotenoids coupled to lipoxygenase also operate in a similar co-oxidant role.

Anti-oxidants

Ascorbic acid, tocopherol, flavonoids and carotenoids all have an antioxidant capacity and are capable of mediating the rate of oxidation of key chemicals. Ascorbic acid is of particular interest because of its dual role in acting as a competitive substrate for endogenous peroxidases and as a substrate for ascorbic acid oxidase as well as a chemical antioxidant. In respiring plant tissue, ascorbic acid is regenerated from the oxidized dehydro form, by an active enzymic system and allows the maintenance of this protection system. In post harvest tissue, many of the protection systems can not be maintained allowing the oxidation of vulnerable species including key quality chemicals.

Pro-oxidants

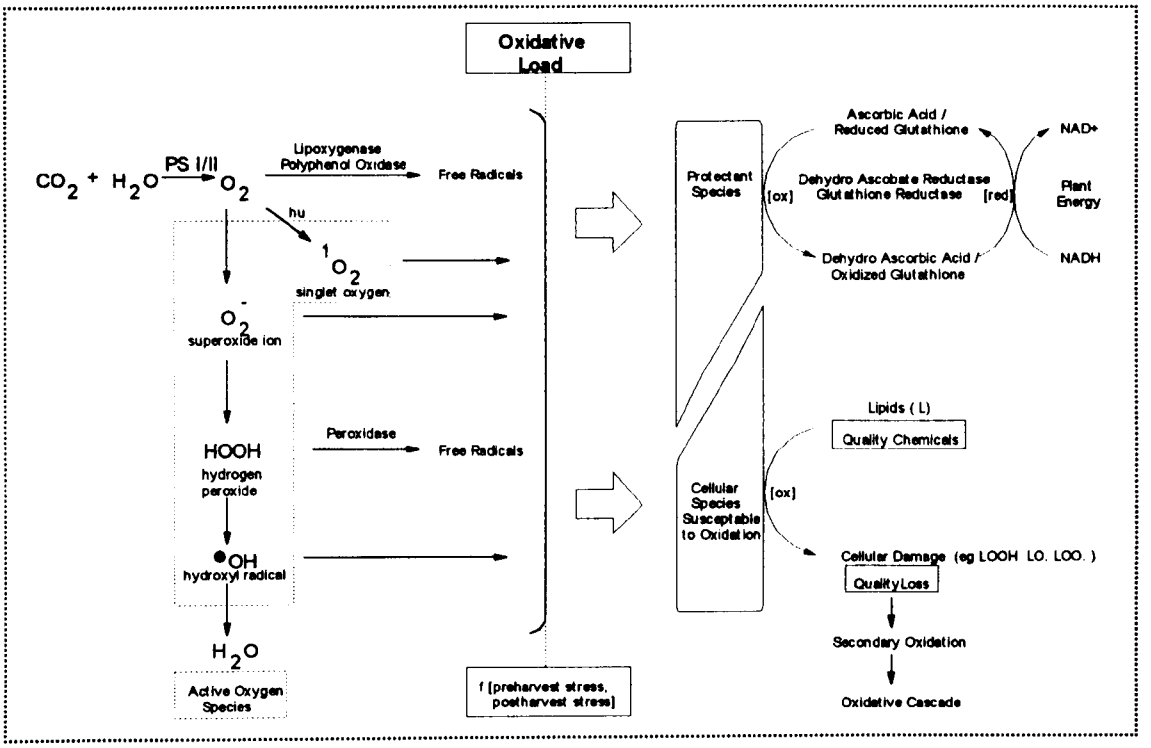
Transition metal ions (eg. iron, copper) are involved in many free radical reactions and often lead to the generation of very reactive species from less reactive ones (Halliwell *et al.* 1989b). The concentration of an antioxidant and its environment can be critical in terms of its effectiveness. Kanner *et al.* (1977) showed the effect of ascorbic acid/ Cu^{2+} ion concentration and water activity on carotenoid destruction. At relatively high concentrations of ascorbic acid in the presence of Cu^{2+} ion, a significant antioxidant effect was achieved. However a substantial prooxidant effect was observed at low ascorbic acid concentration with or without Cu^{2+} ions.

Active Oxygen Species

Endogenous active oxygen species (AOS), produced by the reduction of molecular oxygen, form an important part of the mechanisms by which plants detoxify invading organisms and chemicals, but stray AOS also initiate undesirable oxidation of important cellular chemicals (Figure 1.13; Foote 1985; Halliwell *et al.* 1989a). In healthy plant tissue natural

protectant systems maintain the oxygen toxicity balance however certain pre-harvest and post-harvest stress can disrupt this balance resulting in oxidative damage (Halliwell *et al.* 1989b).

Figure 1.13 Active Oxygen Species and Oxidative Load in Plants



In parsley *(in-vitro)* singlet oxygen and hydrogen peroxide have been highlighted in menthatriene degradation, and peroxidase mediated degradation of chlorophyll respectively. Dihydrofurmaric acid, like ascorbic acid, is a powerful reducing agent and may also be involved in the generation of superoxide ion and the further reduction to hydrogen peroxide (AOS; Figure 1.13). The production of catalytic amounts of hydrogen peroxide, required as the co-substrate for peroxidase action, is discussed in Section 1.6.5. Active oxygen species are inherently difficult to monitor, due to their transient nature. Monitoring generally involves using 'spin traps' under *in-vitro* conditions although recently more valuable *in-vivo* methods have been developed.

Photosensitizers

Photosensitizers are species which, following radiation, react with oxygen to generate singlet oxygen. In parsley a number of well established photosensitizers exist, for example the furocoumarins (Ceska *et al.* 1987) and the porphyrins.

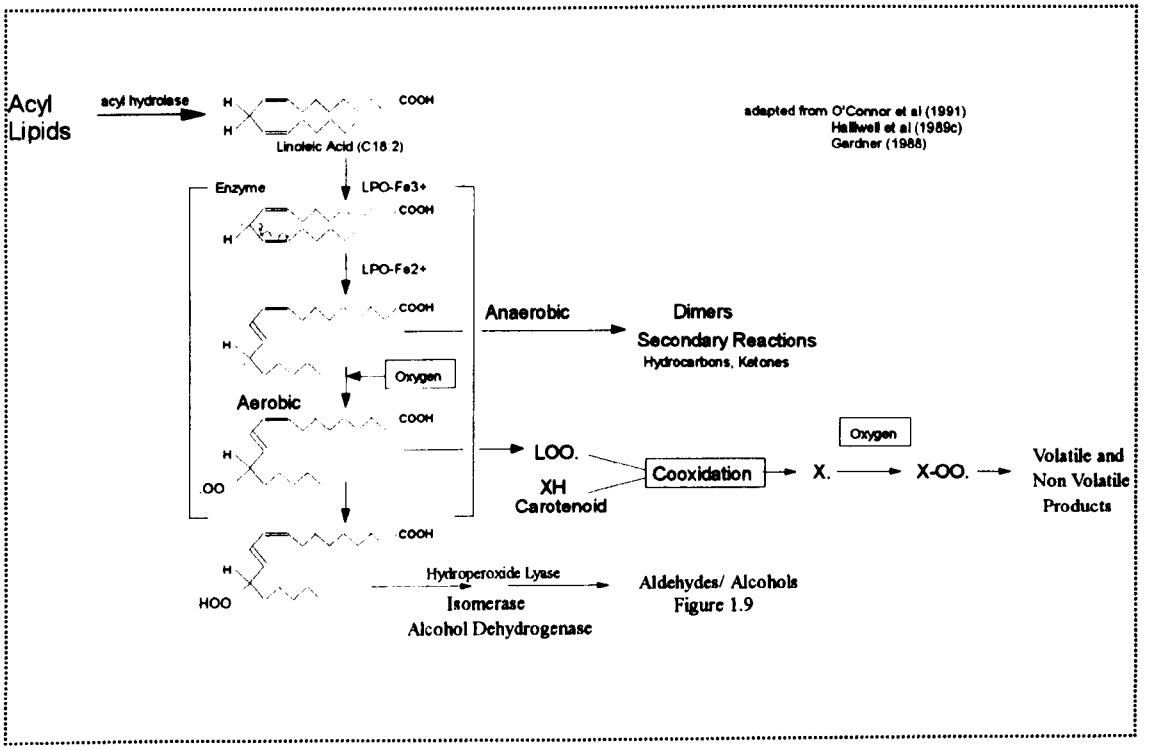
1.6.5 Oxidoreductase Enzymes.

The oxidoreductase enzymes, including lipoxygenase, polyphenol oxidase and peroxidase, are a group of enzymes which have a specific physiological role within plant tissue, as well as influencing the flavour quality of post harvest tissue. In this section the mode of action for each enzyme is summarized to provide an interpretive base for the post harvest dynamics of endogenous chemicals discussed later in this thesis.

(i) Lipoxygenase

The action of lipoxygenase, often initiated by cell damage, catalyses the oxidation of specific substrates containing a cis,cis,1,4 pentadiene moiety, with the consumption of oxygen, to produce the corresponding hydroperoxide. Typically unsaturated fatty acids, liberated by the action of lipase on polar lipids, form the substrate and produce the hydroperoxides which react further to generate volatile and non volatile compounds through biochemical and chemical reactions. The key stages of the mechanism show the binding of the fatty acid to the enzyme, the abstraction of a doubly allylic hydrogen and the subsequent radical delocalisation and double bond shift. Oxygen is then incorporated to form the peroxy radical which abstracts a hydrogen and is dissociated from the enzyme as the hydroperoxide, (Figure 1.14; O'Connor *et al.* 1991).

Figure 1.14 Reaction Mechanism for Lipoxygenase



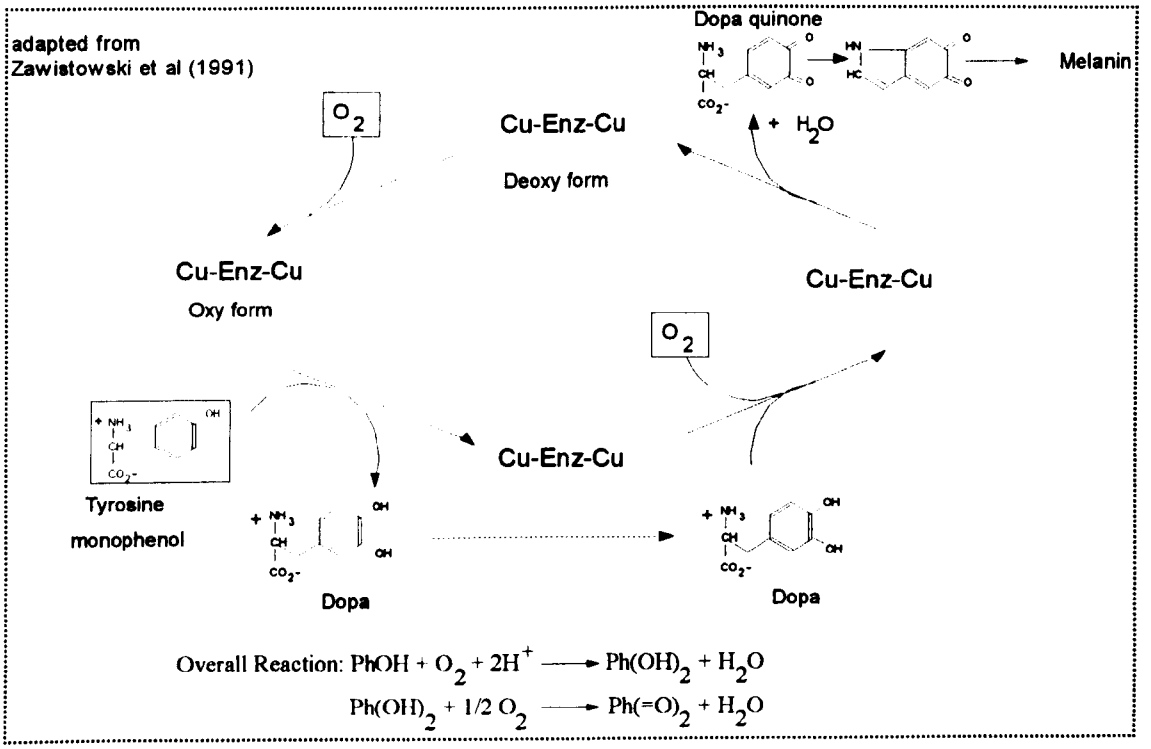
Under anaerobic conditions, lipoxygenase can potentially operate by a modified mechanism where the lipid radical dissociates from the enzyme prior to the insertion of oxygen, generating a range of volatile and non volatile products through chemical reactions (Gardner 1988).

In addition to an aerobic and anaerobic mode, lipoxygenase can also operate via a co-oxidation mode. Halliwell *et al.* (1989c) proposed a sequence whereby the peroxy radical associated to the enzyme is released or partially released to permit the oxidative abstraction of a hydrogen from an adjacent carotenoid (Figure 1.14). Stevens (1970), investigated the role of lipoxygenase in a co-oxidative role in tomatoes to show how various carotenoids break down, presumably through the hydroperoxide, to yield a range of volatile ketones including citral and β-ionone.

(ii) Polyphenol Oxidase

The action of poly phenol oxidase, catalyses the oxidation of certain monophenols, with the consumption of oxygen, to the ortho diphenol and in turn to the ortho quinone (Figure 1.15; Zawistowski *et al.* 1991).

Figure 1.15 Reaction Mechanism for Polyphenol Oxidase

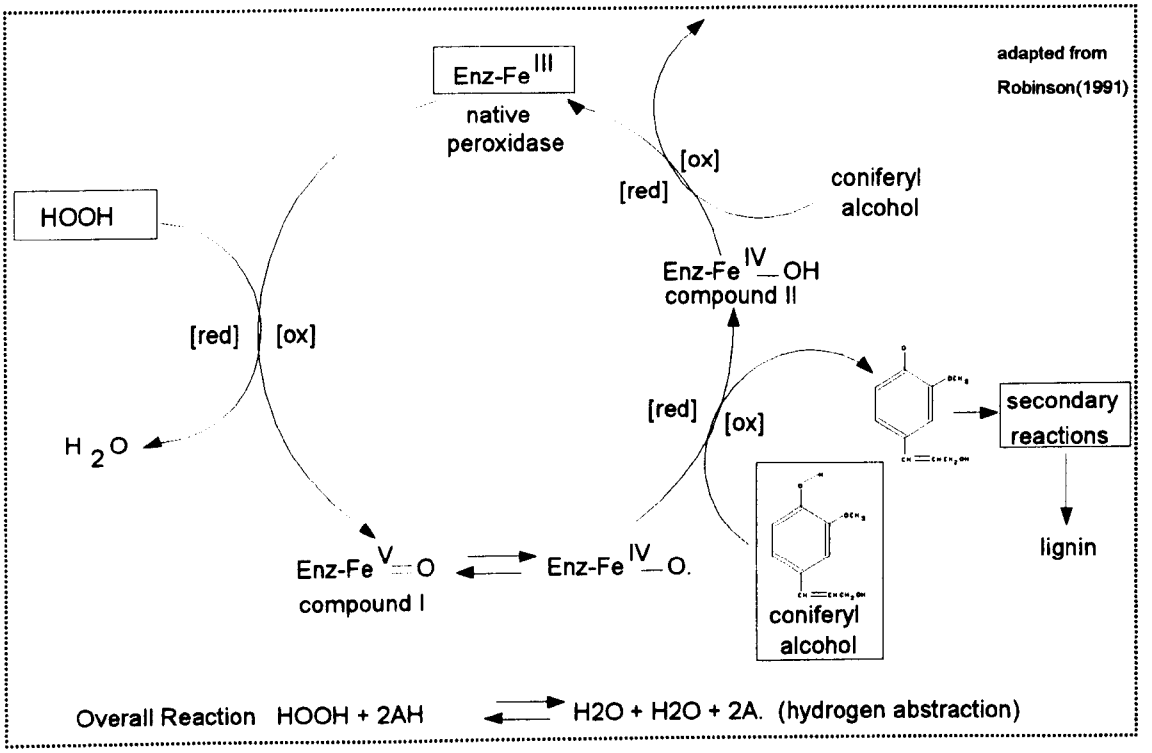


Ortho quinones are reactive species and undergo chemical polymerization reactions, to form pigmented polymers responsible for tissue browning, as exemplified by the conversion of dopa quinone, formed by the action of polyphenol oxidase on tyrosine, to melanin (Figure 1.15; Mason 1948).

(iii) Peroxidase

In the above two examples of oxidoreductases, lipoxygenase and polyphenol oxidase, oxygen is reduced as the co-substrate, whilst the respective substrates, fatty acid and monophenol are oxidized leading to relatively specific initial products. For peroxidase the co-substrate can be hydrogen peroxide or oxygen, depending on the mode of action under which peroxidase operates and this leads to a variety of free radical products. The free radicals formed undergo a complex range of oxidative chemical reactions involving various non-specific species. Although peroxidase is identified as an important enzyme in affecting flavour quality, unlike lipoxygenase and polyphenol oxidase, it's specific role against individual quality chemicals is largely unknown.

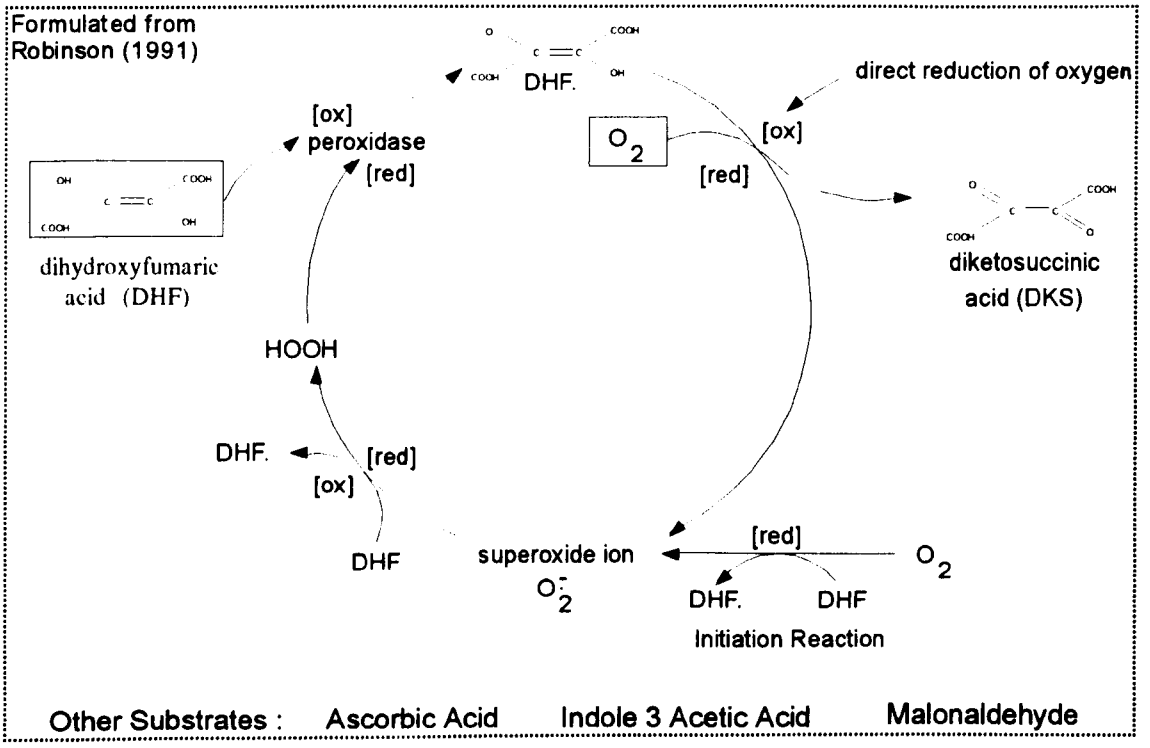
Figure 1.16 Reaction Mechanism for Peroxidase (Peroxidatic Reaction)



The normal mode of action of peroxidase is the peroxidation mode whereby hydrogen

peroxide is reduced by the enzyme. The resultant oxidized form of the enzyme regenerates its initial oxidation state by the sequential removal of two hydrogens from a suitable substrate/s causing their oxidation. Peroxidase is relatively specific with respect to the co-substrate, HOOH, however it is non specific towards phenolic substrates where the reactive phenoxy radical is generated. This undergoes a variety of secondary reactions producing quinones, dimers and alkyl radicals, which react further, (Figure 1.16). Peroxidase can also operate in an oxidation mode against selected substrates with the ability to reduce molecular oxygen to the superoxide ion. The superoxide ion in turn regenerates hydrogen peroxide and provides a cyclic pathway where only catalytic amounts of hydrogen peroxide are required with molecular oxygen appearing as the primary co-substrate consumed, (Figure 1.17). The final and most non specific mode of action is the hydroxylation mode where the native peroxidase can react with a superoxide ion to generate the extremely reactive hydroxyl radical. These radicals readily add to carbon double bonds initiating further radical based reactions.

Figure 1.17 Reaction Mechanism for Peroxidase (Oxidatic Reaction)



1.7 Review Summary

Within this review, flavour quality has been defined as a combination of aroma, taste, colour and texture. These attributes are delivered from plant tissue via a complex and specific range of chemical species present in the food and are subject, through chemical and biochemical processes, to change and manipulation through raw material selection and the processing/storage regimes employed (Figure 1.1). Frozen unblanched parsley has been selected as a suitable tissue for investigation because of the significant colour and aroma quality deterioration displayed under typical commercial frozen storage conditions. Colour quality loss is linked to chlorophyll degradation with the possible involvement of the peroxidase/flavonoid oxidative couple. Aroma quality change has been reported to relate to a loss in 'freshness' and 'grassy' character and an increase in 'haylike' off-flavour component although little information is available on the aroma chemicals responsible for change. Total aroma volatiles have been reported to decrease on frozen storage however no quantitative information on the individual species responsible is available. Menthatriene, a major parsley volatile, is cited to decrease on frozen storage with the appearance of the associated 1,4 endoperoxide. *In vitro* studies have established a mechanism involving the 1,4 addition of singlet oxygen; however it's role *in vivo* under dark storage conditions remains unclear.

In a review of the volatile chemicals reported in parsley, 105 components were identified and a computer database established to record relevant properties including structural information. From the volatile and non volatile components identified, five groups have been defined to allow the most relevant chemicals related to quality, to be categorized (Table 1.4):-

(I) components observed to change during technological processing of parsley.

(II) key odour potent/quality potent components in parsley.

(III) components which represent a particular chemical class/route of formation and are of significance due to their odour threshold/gravimetric standing

(IV) secondary species associated with quality chemical change.

(IV) enzymes associated with quality chemical change.

The chemistry and biochemistry of these chemicals have been reviewed in detail with respect to the routes of formation and loss, relevant to parsley, and have identified the importance of oxidative processes. From this review, it is apparent that endogenous enzymes have a role to play in determining the flavour quality under typical post harvest processing regimes. Of particular interest is the potential role of peroxidase in forming an oxidative couple with endogenous phenolic compounds capable of the further oxidation of endogenous compounds. These enzymic changes operate in parallel and even overlap with similar chemical processes to the point where the contribution from each source is unclear and will need to be resolved if the roles of enzymes are to be put in context. Equally important is the realization of the lability of the volatiles, pigments and taste chemicals, and their sensitivity towards the work up procedures during analysis, consideration of which will be central to the analytical strategy of this programme.

1.8 Aims and Approach

The aim of this thesis is to investigate the chemical and biochemical mechanisms responsible for flavour quality change in frozen unblanched parsley, and to use the information to extend the understanding of the flavour quality and shelf-life of vegetables and herbs.

In this thesis, novel methodology will be developed, as part of a unified analytical approach, to monitor volatile and non volatile species from a single plant tissue sample in order to facilitate the correlation between transient species. This approach will be quantitative and focus on minimising artifactual change through analytical extraction, separation and detection (Chapter 3).

Application of this method to establish the dynamics of key quality chemicals and related species as a function of controlled processing stress will be undertaken (Chapter 4).

Secondary experimentation, designed to establish the respective roles of chemical and biochemical processes in the observed changes, using thermal inactivation of enzymes and the control of oxygen, will be performed (Chapter 5).

Finally, from the information generated, model systems will be established to investigate further the nature of the chemical and biochemical mechanisms relevant to frozen parsley (Chapter 6).

Chapter 2

Materials and Methods

2.0 Materials

Material	Grade	Supplier
Plant Tissue		
Parsley seed 'Smaragd'	1992,1993,1994	Langnese-Iglo Reken
Sweet Marjoram	1992 plants	local nursery
Spinach		local market
Cabbage		local market

Material	Grade (Code)	Supplier
SNCVA / SNCNVA		
Chloroform	HPLC	Rathburn Chemicals Ltd.
Methanol	HPLC	Rathburn Chemicals Ltd.
Ethyl Acetate	HPLC	Rathburn Chemicals Ltd.
Acetone	HPLC	Rathburn Chemicals Ltd.
Formic Acid	Sequencer	Rathburn Chemicals Ltd.
Water	Grade A Milli Q	Millipore (UK) Ltd.
α-Tocopherol	Biochemika (95240)	Fluka Chemicals.
Ascorbic acid	ACS 98% (A1417)	Sigma-Aldrich Co.
p-Coumaric acid	Purum 98% (28200)	Fluka Chemicals.
Apigenin	HPLC std (7002)	Roth Chemicals.
Apigenin-7-glucoside	HPLC std (5557.1)	Roth Chemicals.
Hexanal	98% (11,560-6)	Sigma-Aldrich Co.
trans-2-Hexenal	99% (13,265-9)	Sigma-Aldrich Co.
Parsley Leaf Oil	Food (FEMA 2836)	R.C.Treatt.
Menthatriene	commissioned supply	R.C.Treatt.
p-Cymenene	Flavour (965)	Bedoukian Research Inc.
GLC Test Mix 31	GLC	Chrompack Ltd.
Phosphoric acid (85%)	Biochemika (79617)	Fluka Chemicals.
Sodium dihydrogen phosphate	Ultra 99% (S8282)	Sigma-Aldrich Co.
Disodium hydrogen phosphate	Ultra 99% (S7907)	Sigma-Aldrich Co.
Tetra Butyl Ammonium Phosphate	Puriss (86899)	Fluka Chemicals.

Material	Grade	Supplier
Enzyme Activty Assay / Enzyme Systems		
Peroxidase (Horseradish) EC 1.11.1.7	Type II MW=44,000	Sigma-Aldrich Co.
Polyphenol Oxidase EC 1.14.18.1	Tyrosinase (T7755)	Sigma-Aldrich Co.
Hydrogen Peroxide	30% soln (H1009)	Sigma-Aldrich Co.
Naringenin	HPLC std (4199)	Roth Chemicals.
Umbelliferone	Purum 98% (93979)	Fluka Chemicals.
Catechol	99% (C9510)	Sigma-Aldrich Co.
Catechin	99% (C1251)	Sigma-Aldrich Co.
Guaiacol	Purum 98% (50880)	Fluka Chemicals.
Chlorophyll 'a'	Spinach Chl 'b' free	Sigma-Aldrich Co.
Lycopene	HPLC std (5670.1)	Roth Chemicals.
Dimethyl sulphoxide	Puriss 99% (41648)	Fluka Chemicals.
3-Methyl-2-benzothiazolinone hydrazone hydrochloride	Purum 99% (65875)	Fluka Chemicals.
3-(Dimethylamino)benzoic acid	97% (D0787)	Sigma-Aldrich Co.
Triton X100 (reduced)	Peroxide free	Sigma-Aldrich Co.
Sodium chloride	Microselect (71378)	Fluka Chemicals.
Potassium chloride	AR	Sherman Chemicals.
Sodium Hydrosulfite	84% (S1256)	Sigma-Aldrich Co.

2.1 Methods Derived from the Literature

2.1.1 Parsley Cultivation and Frozen Storage Protocol.

'Smaragd' *Petroselinum crispum* was cultivated under glass (1992, 1993 harvest) and in the field (1993, 1994 harvest) with nutrient monitoring (water, light) and defined growing compost. Plants were grown in 6" unit cells, arranged in a 6 x 60 unit cell matrix. At harvest, the stems, along with the associated leaf canopy, were individually cut at the root base and inverted into liquid nitrogen contained in a large stainless steel dewar flask. Leaflets were removed from the stems by making a cut below the level of the liquid nitrogen. The dewar flask was repeatedly stirred to reduce the size of the frozen leaflets and to ensure good homogeneity of tissue within the dewar. Each dewar flask, containing 12 plants, was transferred to a -30°C freezer room and under the appropriate safety protocols, strained and distributed evenly between 12 glass 1 litre Kilner jars and sealed using PTFE seals. Jars (x2) were transferred for storage at -65°C , the remainder held at the storage temperature under investigation (-10°C, -20°C) for the required time period before being re-transferred to -65°C, prior to assay. Sweet marjoram, *Majorana hortensis*, was purchased as mature plants from a local nursery and processed as described for parsley.

2.1.2 Peroxidase Extraction Protocol.

Frozen parsley (5g) was homogenized (30,000 rpm, 10 minutes) into cooled 80 mM pH 6.5 phosphate buffer containing 1.0 M sodium chloride (20 ml, 0°C). The mixture was centrifuged (4,500 rpm, 5 minutes), and the translucent liquor removed, by syringe, for peroxidase activity assay.

2.1.3 Peroxidase Activity Assay (Ngo *et al.* 1980).

An aliquot of peroxidase test solution (25µl) was added to 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH) solution (70 µM in 80 mM pH6.5 phosphate buffer; 5ml) within a stirred reaction vessel (10ml). Two cuvettes containing distilled water (1ml)

were preloaded onto a multi cuvette carriage (reference and sample position 1) within a UV-visible spectrophotometer (Shimadzu UV-2101PC), and monitored at 590nm. A third empty cuvette was preloaded into 'sample position 2'. 3-(Dimethylamino)benzoic acid (DMAB) solution (660 μM in 80 mM pH6.5 phosphate buffer) containing 2.0 mM hydrogen peroxide (5ml) was added to the stirred reaction mixture and data acquisition, for the previously loaded reference samples, initiated. After 20 seconds an aliquot of the reaction mix (1ml) was transferred to the third preloaded cuvette (sample position 2) and the spectrophotometer multi cuvette carriage switched from position 1 to position 2, and monitored for 300 seconds.

2.1.4 Steam Distillation Extraction (Likens *et al.* 1964).

Frozen leaves (50 g) were transferred to a reflux vessel (500 ml) containing distilled water (300g). The mixture was refluxed for 2 hours using pentane: diethyl ether (15g 1:1) as extraction solvent. After reflux an aliquot of solvent (~0.5 g) was diluted with chloroform (20ml) and methanol (10ml) and injected (1μl) using the GLC component of the SNCVA technique.

2.1.5 Oxygen Electrode Measurement.

Component List

Silicon rubber annular membrane (thickness 1/1000", circumference 23 mm/50 mm).

PTFE membrane (thickness 1/1000", circumference 60 mm).

Lens tissue disc (circumference 20 mm).

'O' ring (9 mm).

Perspex electrode base (Rank Brothers Ltd).

Perspex sampling assembly (Rank Brothers Ltd).

Glass jacketed measuring cell (Rank Brothers Ltd).

Digital control unit (Rank Brothers Ltd).

Magnetic stirrer flea (glass).

240 Ω calibration resistor.

Electrode Assembly

The silicon rubber annular membrane was placed on the perspex electrode base and the lens tissue disc positioned centrally over the platinum electrode (Figure 2.1). Potassium chloride solution (3 M, 30 µl) was pipetted into the electrode base well and the PTFE membrane (preformed) overlayed and taped into position. The sealing 'O' ring was placed over the platinum electrode and the glass measuring cell lowered into position and clamped. Water (Grade A, 4 ml) was pipetted into the measuring cell and allowed to equilibrate at 20°C with stirring.

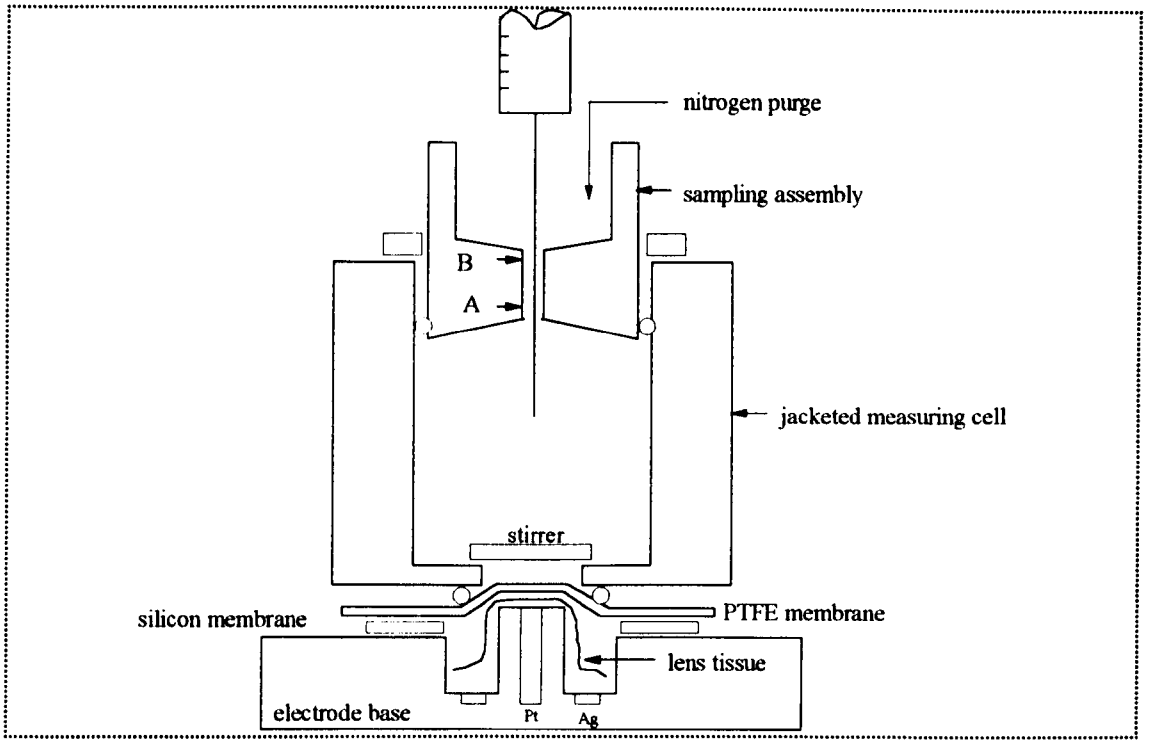
Electrode Calibration

The electrode base was unplugged (3 pin DIN) from the digital control unit and the calibration resistor inserted across the terminals of the current circuit (pin 1 & 3). The sensitivity potentiometer was adjusted to produce an output voltage of 900 mV and the polarizing voltage set to 0.600 V ±0.001. The electrode base was reconnected to the control unit and allowed to stabilize (20°C, 30-45 min with stirring), the output voltage was measured and related to an equivalent oxygen concentration for pure water at 20°C (6.4 cm^3 O_2 . l^{-1}; Kaye *et al.* 1966; voltage values outside the range 900 mV ± 50 required the electrode to be reassembled). Sodium hydrosulfite crystals were added to the water sample and the output voltage measured and related to a zero oxygen level (voltage values outside the range 0.00 mV ± 0.01 required the electrode to be reassembled).

Oxygen Monitoring in Model Enzyme Systems

The measuring cell was cleaned in-situ, (following any previous analysis/calibration), with sequential washing using water aliquots and the final wash volume removed from the cell. Phosphate buffer and the other components of the model system were pipetted into the cell (see Chapter 6, Reagent Tables 1-3) and the electrode allow to stabilize (open to atmosphere with stirring). The perspex sampling assembly was inserted into the top of the measuring cell and the sample meniscus level adjusted to position A (Figure 2.1). The nitrogen purge supply was positioned into the well of the sampling assembly and data acquisition initiated (recording stable plateau). Hydrogen peroxide solution (or polyphenol oxidase solution) was introduced into the measuring cell (to initiate reaction) through the sampling port using a 20 µl microsyringe and the level adjusted to position B (if necessary), by rotating the sample assembly screw collar.

Figure 2.1 Oxygen Electrode (Rank Brothers Ltd)



2.2 Methods Developed within this Thesis

2.2.1 Tissue Solubilisation Protocol.

Frozen parsley (~1.5g, -65°C) was transferred to a pre-weighed amber glass anti vortex vial (40 ml) containing methanol (10ml, -65°C), within a cryogenic box. The vial was re-weighed and transferred to an acetone/ solid $C0_2$ cryogenic mixture. A precooled homogenizer head (Polytron 12 mm type, -65°C) was positioned into the vial, through a sealing septum and the mixture sheared at 30,000 rpm (10 minutes). An aliquot of chloroform (10ml) was added and sheared for a further 5 minutes. A second aliquot of chloroform (10ml) was added and the vial sealed and transferred to a water bath (20°C, 5 minutes). The vial was centrifuged at 3,000rpm (5 minutes) and the extract filtered (0.25μm syringe filter) into two amber autosampler vials.

2.2.2 Stabilized Non Concentration Volatile Analysis Protocol.

Parsley extract (1 µl SNCVA I; 2 µl SNCVA II) was analysed by Gas Liquid Chromatography using the SNCVA protocol (Chapter 3.2.2).

2.2.3 Stabilized Non Concentration Non Volatile Analysis Protocol.

Parsley extract (2 x 50 µl, 10 µl) was analysed by C_{18} reversed phase High Performance Liquid Chromatography using the SNCNVA protocol (Chapter 3.2.3).

Chapter 3

Development and Characterization of Analytical Methods

3.0 Introduction and Review of Analytical Procedures

In Chapter 1, a review of the analytes relevant to frozen parsley was presented along with their associated chemistries and biochemistries relating to their formation and loss. In this section, the objective is to consider current methods and methodological approaches for the determination of these chemical species, and to compare and contrast them with our requirements within this study.

The first step in selecting or developing analytical methods is to identify the target analytes of interest and the type of information required, for example whether the analysis is to be qualitative or quantitative, and the degree of chemical information required, ie. structural or stereospecific.

In this study a wide range of volatile and non volatile compounds have been identified, related to the quality of frozen parsley, which form the primary focus for analytical measurement from intact plant tissue (Table 1.4). However, there is always the possibility that other, as yet unknown, compounds may need to be analyzed and a certain amount of flexibility in the analytical system is also desirable. Additionally, many of the targeted compounds are involved in oxidative change and as such are related through a oxidative cascade operating within the parsley tissue. An analytical system which enhances the ability to allow correlations between volatile and non volatile compounds is desirable.

Traditionally, analytical protocols used to assay plant materials are designed for a single analyte or closely related family of analytes. Typically, the chemicals of interest are extracted from the tissue using a specific gas or liquid phase which displays a selective solubility range towards the analytes of interest. To ensure quantitative extraction, some procedures use a cell disruption sequence with multiple extraction, elevated temperatures and extended times. Unfortunately such multi-stage procedures are likely to cause change in the labile analytes. In this project, it is of key importance to avoid change during extraction and analysis so that the dynamic changes in parsley associated to processing conditions (particularly related to oxidative processes) can be clearly seen. In designing an analytical protocol, the variation inherent in biological systems also needs to be

considered. This relates to the sampling protocol to be used to obtain representative samples and thus determines the weight of sample to be extracted and the number of repeat analyses. A traditional analytical approach, utilizing a series of separate methods to quantify a wide range of analytes, required to be correlated, would potentially have limitations based on the number of separate analyses and the associated time of analysis.

From a general analytical perspective, there are two primary areas for consideration, the amount and type of flavour molecules in the food/plant tissue and the rate at which molecules are released from the food. The analysis of aroma volatiles demonstrates many of the analytical considerations relevant to the non-volatile species, with the exception of partitioning into the gas phase, and, as such, provides a good vehicle to discuss further, important criteria in the selection and development of analytical methods.

Aroma chemicals are small organic molecules distributed within the plant cellular matrix, with a wide range of chemical functionalities, leading to a spectrum of physical properties and chemical reactivities. There are two basic types of aroma analysis, total 'in tissue' analysis and headspace analysis, each offering slightly different information and holding a different position/relation with respect to sensory/quality data, and each offering advantages over the other. If we consider plant tissue with respect to volatile flavour we will observe a system which, prior to harvest will, contain free volatile components and non volatile precursors, for example terpene glycosides, in a state of flux depending on the maturity/stage of ripening and its general environment. At the point of harvest (and during subsequent handling/processing) this state of flux is disrupted to varying degrees due to the applied stresses and the new environment, where the rates of existing processes will change and a whole series of additional processes come in play. These processes, reviewed earlier, can be biochemical or chemical, even microbiological, in origin and may alter the volatile composition via:-

(a) volatile generation from non volatile precursors either directly, for example, in the hydrolysis of terpene glycosides or indirectly via an oxidative sequence involving unsaturated lipids.

(b) conversion to another volatile form.

(c) conversion into a non volatile form, eg. increased polarity or polymerization.

The relevance of stress-induced flavour generation is very important, but only when administering stress of the type and magnitude experienced in commercial production/normal usage. Analysis regimes which operate outside this range must be questioned for relevance and artifact content.

In the context of this study, the most fundamental measurement would be total flavour analysis (quantitative) where the amount of each component in a sample was obtained by extraction and direct extract injection. The realization of this type of analysis will be a key component in this study, however it is subject to a number of analytical problems largely stemming from the means of extraction and the ability to select and control the type and level of stress used. It is therefore necessary to know in some depth the chemistry and biochemistry of the system under study before analysis regimes can be applied/designed with confidence. The first and most important component of a total analysis regime is extraction/isolation (Reineccius 1988). This must be quantitative and performed under zero or well controlled relevant stress conditions. The main consideration is in effecting a total transfer of the analyte from its remote location within the complex cellular matrix into the extracting solvent. Here cell disruption is often employed. However many biochemical and chemical reactions are favoured on de-compartmentalization, when endogenous stabilization mechanisms are removed and when access of substrates with reactants/catalysts occurs. For volatiles, an additional constraint is the removal of volatile material from interfering non-volatiles, where the select properties of flavour volatiles are used singly or in combination as the basis of selective extraction, namely their lipophilic and volatile nature. Similarity for non-volatile analysis, other interfering non-volatile species must be removed, again using selective properties of the chemicals involved.

To date we have discussed flavour extraction (sample preparation) as the first component of a total analysis regime. Other components include, (i) introduction of the sample onto the analytical column (injection), (ii) component separation, (iii) detection and (iv) data analysis. Each of these topics are subjects in their own right and only a brief overview of the important points will be highlighted in this text.

Injection methods for quantitative work normally involve cool on-column techniques coupled to some refocusing system, either a retention gap and or cryo-focusing. This

method avoids the problems of high temperature vaporization systems which may form artifacts or cause thermal degradation, although such techniques do have particular applications. Component separation should ideally utilize both polar, non-polar and chiral phase chromatography either separately or using a two dimensional configuration (so-called column switching or heart cutting). Detection systems fit into three categories (i) quantitative (ii) qualitative and (iii) olfactory. The most common quantitative detector is arguably the flame ionisation detector, showing good sensitivity and linear range over a wide spectrum of organic compounds. Qualitative detection generally involves mass spectrometry supported by a series of complementary techniques such as FTIR, NMR, FPD(partially selective) and circular dichroism (CD). Circular dichroism is used to identify stereo isomers and can be used to establish the chemical or enzymic origins of a compound. The third detection system, olfaction, offers unique quality-related information, but requires the use of a sensory language to describe the sensations perceived. Like all languages, different words are used to describe common things resulting in a myriad of descriptors. For many years flavourists, perfumers and psychologists have tried to classify odours. Notably, Harper *et al.* (1968) suggested a scheme based on commonly available standards.

The other basic method referred to in the above text is headspace analysis. This technique holds a unique position in flavour research because of its potentially close links to olfactory/quality measurements. The method, in common with the olfactory process, involves sampling the gas phase above the sample and offers the advantage of little or no sample disturbance/work up, along with no contamination from non-volatiles. The main disadvantage with headspace analysis is that the relationship to total flavour can be complicated, based on vapour pressure and the solution properties of the matrix from which it is released. Additionally the method requires a finite period for the volatile flavour material to transfer into the gas phase. This period is critical for quantitative work and is often further complicated by a sample which is changing, for example enzymic generation of flavour as occurs in many vegetables. The last main limitation is the low concentration of volatiles in the headspace and often a selective concentration step is inevitable. Because of these limitations, a total flavour analysis protocol based on solvent extraction will be used to investigate the chemical status of plant tissue in this study.

3.1 Unified Strategy for the Analysis of Volatile and Non Volatile Components

The chemicals identified at the beginning of this chapter can potentially be included in a unified analysis strategy based on a total 'in-tissue' analysis sequence. This unified analysis strategy for volatile and non volatile species aims to provide a single stabilized solution from plant tissue, as a means to establish the in-tissue concentrations with minimal isolation stress and free from any artifacts. The single solution extract can be subsequently analysed by GLC and HPLC as parallel separation techniques for volatile and non-volatile species respectively. The development of an integrated protocol for extraction, volatile analysis and non-volatile analysis from a single sample is described later in this thesis.

3.1.1 Analyte Extraction and Solubilization

In this section the aim is to develop a suitable approach, by defining the key criteria for the method and detailing the design, implementation, and characterization of such a method.

The primary criteria for this technique are:-

(i) quantitation

(ii) rapid, reproducible measurement of analytes in tissue.

(iii) minimisation of artifacts.

To achieve the quantitation criteria, all the flavour/analyte molecules must ideally be extracted from the tissue. Although numerous extraction techniques are available (Reineccuis 1988), they generally involve partitioning/dilution into the extracting phase and reconcentration to permit chromatographic separation. These processes are often selective and require quantitation in their own right for the vast range of volatiles/analytes to which they are subject. For our analysis, and as a result of the relatively high flavour levels in herbs, it is proposed to use a solution approach, whereby all the volatiles/analytes are solubilized to form a homogeneous solution, which can be subsequently analyzed by a direct on-column GLC/HPLC method, to provide quantitative chromatographic data. The criteria to prevent artifactual change will be based on a 'stabilization concept' using chemical and physical means, and based on a knowledge of likely change, via a

combination of chemical stabilizers/inhibitors and low temperature. The above components of the analysis, solubilization and GLC/HPLC separation, are all inter-connected components and are designed as such and overlaid by the stabilization concept. The technique has been termed Stabilized Non Concentration Volatile Analysis (SNCVA) and Stabilized Non Concentration Non-Volatile Analysis (SNCNVA).

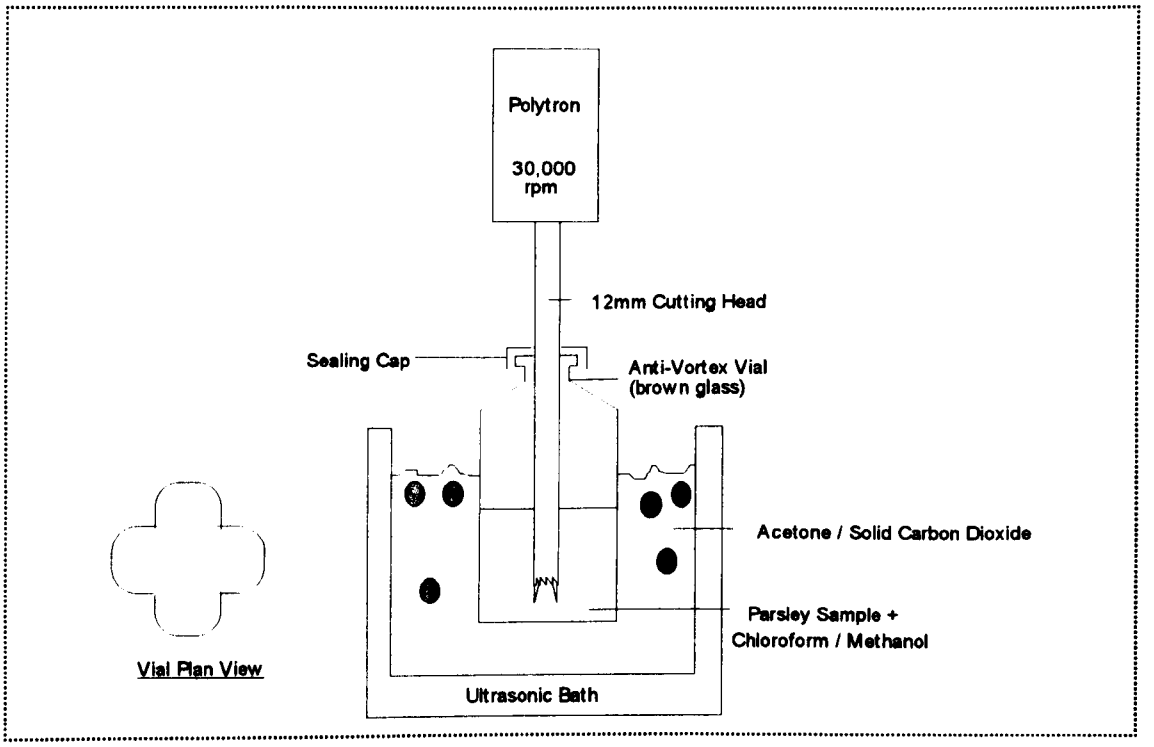
The primary consideration in developing a solution method is that all the macroscopic phases and cellular structures into which flavour analytes can partition/associate are solubilized, along with the flavour analytes themself. This will lead to a situation whereby only inert particulate material remains in the extracting liquor which can be removed by physical means. Two potential solubilization approaches are available, homogeneous/true solution or heterogenous/colloidal solution. The first might involve a broad spectrum solvent system using chloroform/methanol (Folch *et al.* 1957), and is the option favoured and developed here. The second option is a reverse micelle system using Aerosol OT/cetyl alcohol, similar to that used in the dry cleaning industry. Under either regime, the sampling volume for GLC/HPLC separation is sufficiently large, (> 1µl), so that both will be quantitatively sampled. If we consider parsley tissue in terms of the type and level of chemical species present and the form in which they are organized within the cellular matrix, the requirement for the solubilization system can be established. The main non polymeric load for the solvent system is likely to be materials at the limits of the polarity scale, including water and the lipids/hydrocarbons respectively. In both the homogeneous and heterogeneous solution modes, water at >80%w/w is likely to be the controlling entity, as chloroform/methanol and the interior of the reverse micelle has a finite capacity for water. In addition to the above non polymeric load, plant tissue also contains a significant polymeric load. The logic applied to this material is that it is largely hydrophilic and therefore not a sink for lipophilic flavour and can be removed as a particulate. Some polymers however are lipophilic, for example, cutin, responsible for the structural element of the cuticle layer overlying epidermal cells, and is potentially an issue in this respect. Although the intracuticular waxes can be effectively extracted from the cuticle, the question remains whether this polymer can act as a significant sink for flavour when in contact with extracting solvent. This question will be experimentally tested as part of the characterization sequence.

With all extraction methods it is important to ensure intimate contact between the extractate and the extractant, and this is particularly true for plant tissue as most of the target species form part of organized cellular and sub cellular structures. A number of cell disruption methods are available, most employing physical processes ie high shear, cutting, freeze/thaw and ultrasonics however certain enzymic processes have also been used (Keshavarz *et al.* 1987). Enzymic digestion has been applied specifically to parsley with a relatively high degree of success with 95% disruption into single cells (Zetelaki-Horvath *et al.* 1977). Interestingly the author of the work comments that, after treatment, the taste and flavour appeared to be unchanged, although no data were given in support. The key issue in all these treatment is the type and degree of chemical/biochemical change induced, and strategies must be developed to minimise these. If we consider the likely processes relevant to parsley based on the earlier literature review we can identify suitable stabilizers to permit the desired control. An important consideration when proposing the addition of a stabilizer is that it should not interfere with endogenous materials at the later separation phase, ie co-elute with a target analyte, or create a interfering process in its own right.

The stabilization protocol must first inhibit all enzymic processes. In our system, methanol has been selected because of its dual role of enzyme inhibitor and as part of the solvent system. Significantly the methanol/chloroform solvent system allows very low temperatures to be maintained throughout the procedure, typically -65°C, and the capability of dissolving ice and other crystalline lipid phases is important in preventing analytes being locked up in solid matrices. Typical enzyme inhibitors like trichloroacetic acid are not deemed appropriate because of their acidic properties. Autoxidative change can be controlled using free radical scavengers, for example tocopherol or tert-butyl hydroquinone (TBQA). The action of these materials is also directed at the chloroform component of the solvent system as this has a tendency to form the oxidising $CCl_3\bullet$ radical, where α-tocopherol is particularly effective (Simic 1985). When selecting an antioxidant, considerable care must be taken to ensure the system chosen is tailored to the environment in which is its designed to be operative, for example lipid based antioxidants can have a negative effect in aqueous systems (Aruoma 1993). Photoxidative change can be controlled using 1,4 diazabicyclo[2.2.2]octane (DABCO) and by conducting the whole procedure in amber glass and low light conditions. Metal catalysed reactions are

potentially stabilized by using the chelating properties of EDTA although it is important to select forms of EDTA with a neutral pH. However, as for antioxidants, considerable care must be taken to ensure the required effect. Additional factors for consideration are the endogenous stabilization systems which are disrupted, for example ascorbic acid and Cu^{2+} ions form a couple which can act as a proxidant depending on the concentration (Kanner *et al.* 1977). In a study on the effect of aromatic herbs on the autoxidation of sardine muscle and oil, parsley was shown to have a proxidative capacity (Pizzocaro *et al.* 1985). Finally, to minimise acid catalysed reactions the use of a buffer system should be considered. However the role of pH in a chloroform/methanol/water system needs to be considered further. Development and characterisation of the extraction/stabilization procedure (Figure 3.1) is discussed within the experimental and results sections of this chapter.

Figure 3.1 Stabilized Non Concentration Analysis - Extraction



3.1.2 Stabilized Non Concentration Volatile Analysis (SNCVA)

'SNCVA I'

The SNCVA technique is a concerted protocol involving solubilization/extraction and chromatographic separation. The objective of this section is to consider the development

and characterisation of the separation of the volatiles within the extraction liquor. In the development sequence, two forms of the SNCVA technique (*SNCVA I and II*) were sequentially developed, using the same underlying principles, but implemented using a slightly different chromatographic arrangement.

The primary criteria for extract analysis are as follows:

(i) quantitation

(ii) removal of interfering non volatiles

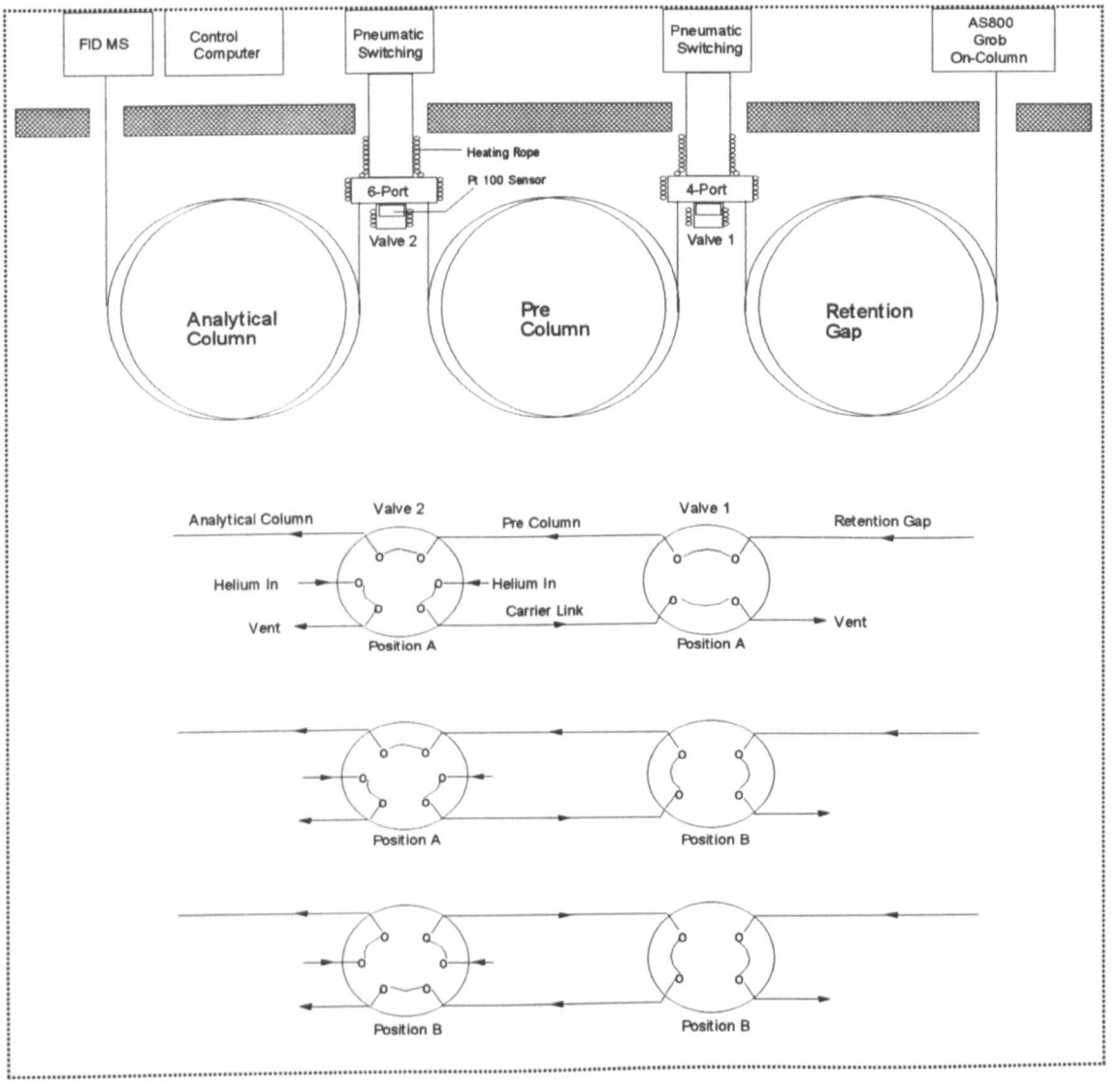
(iii) removal of low volatility material poorly eluted from the analytical column

(iv) provide volatile focusing to produce good separation

(v) provide an environment to minimise chemical change.

To meet the above criteria, a liquid injection procedure, using a Grob on-column injector coupled to a retention gap, pre-column and analytical column, via two switching valves, was designed and constructed (Figure 3.2). The analytical sequence involved making a liquid injection on to the retention gap with carrier gas flow directed towards the pre-column/analytical column (valve 1 @ position A, valve 2 @ position A). After volatile transfer the 4 port valve was switched to allow flow from the retention gap to be directed to vent whilst separation continued within the pre-column/analytical column (valve 1 @ position B). Repeated solvent injections were made to allow liquid washing of the retention gap followed by a helium flow to condition the sample path. As the oven temperature increases, under program control, the pre-column was back flushed to prevent the low volatility material transferring onto the analytical column (valve 2 @ position B). Liquid injection provides the most quantitative means to transfer a sample for GLC separation and eliminates the high reactive temperature of vaporization injectors. By utilizing a retention gap the volatiles are concentrated into a suitably tight band for GLC separation whilst the non-volatile material, including the chemical stabilizers, are retained. This allows extraction and concentration to be performed on-line, eliminating volatile loss, with the stabilizers functioning until the point the volatiles enter the inert carrier gas phase. To overcome the problem of partial transfer of low volatility material onto the analytical column, a pre-column has been used. By back flushing this column, the components which would require high temperatures and long times to elute are removed allowing reduced column conditioning and improved column reproducibility.

Figure 3.2 Stabilized Non Concentration Volatile Analysis I (SNCVA I)



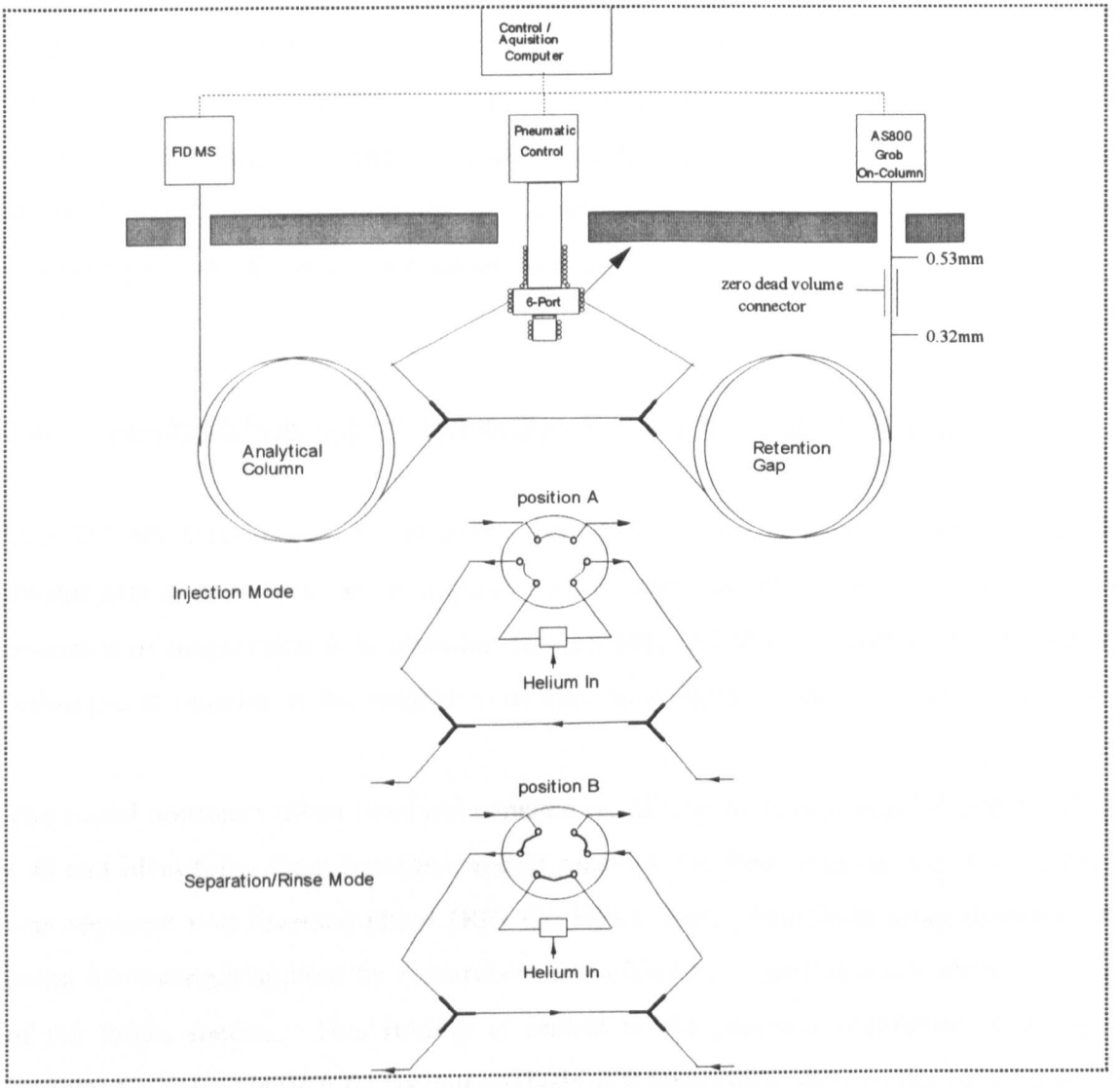
'SNCVA II

As a result of the development work on SNCVA I, presented in Section 3.2, two regions within a typical chromatogram were identified which required further development. At low retention times the solvent tail interferes with the elution of the volatile products of lipoxygenase action (eg hexanal) and, equally the size and shape of these peaks is not ideal with respect to quantitation and qualitation. The second area for development was at longer retention times where variability in the peak area of myristicin was observed.

The poor dynamics of the solvent peak are believed to be associated with an inherent incompatibility between the chlorinated solvents used and the chlorinated polymer rotor

material within the Valco valves. Few options are available with respect to changing this material as certain temperature requirements must be maintained (ie programmed oven temperature ~ 200°C). An alternative to improving the solvent tailing phenomenon in relation to hexanal quantitation, would be to increase the polarity of the analytical column phase to permit greater retention of these aldehydes and alcohols. However, the solvent peak is also likely to interact further and may still partially mask other components of interest.

Figure 3.3 Stabilized Non Concentration Volatile Analysis II (SNCVA II)



To resolve this phenomenon the system was redesigned, maintaining all the key criteria and components detailed in SNCVA I, with the exception of the analytical precolumn, by isolating the valves from the chromatographic path. This was achieved using pressure

switching across two connected zero dead volume 'press fit' tee connectors (Figure 3.3). In this system, carrier gas flow entered the chromatographic path via the retention gap (0.6 ml/min) and at reduced flows (0.2 ml/min), along the inlet arm of each tee, such that there was a positive flow transferring the injected sample from the retention gap through the transfer line onto the analytical column. After a set period the 6 port valve was switched, to back flush the transfer line and allow the retention gap to be liquid flushed. In this configuration, carrier gas flow exits the vent arm via the first tee allowing liquid washing of the retention gap whilst the pressure regulated carrier gas supply to the second tee provided separation flow for the analytical column and backflush flow for the transfer line. In this sequence sample/rinse injections and valve switching are automated via a Fisons Instrument AS800 autosampler and Valco pneumatic actuator respectively and controlled by a Fisons Minichrom data acquisition/control computer. This automated protocol allowed improved reproducibility and confidence of data, as well as significantly increasing the sample analysis capacity with overnight operation.

3.1.3 Stabilized Non Concentration Non Volatile Analysis (SNCNVA)

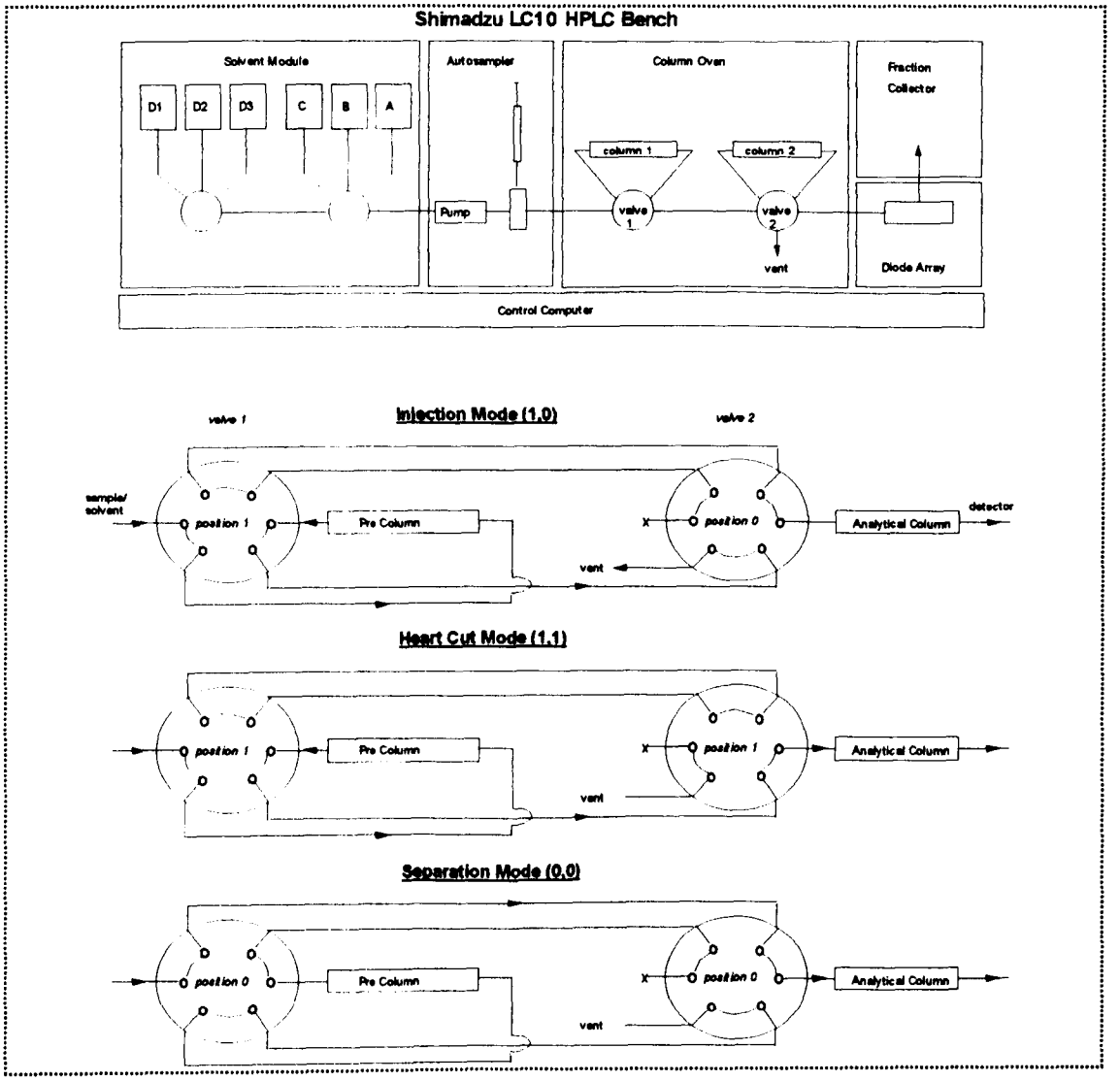
The SNCNVA technique, in common with SNCVA, is a concerted protocol involving solubilization/extraction, as discussed earlier, and chromatographic separation. The objective of this section is to consider development and characterisation of the proposed technique in relation to the separation of the non-volatiles within the extraction liquor.

The initial approach taken involved considering all the analyte groups of interest (Table 1.4) and identifying from literature, recent methods for their analysis. From these data it was apparent that reversed phase (RP) C_{18} HPLC with photodiode array detection was being increasingly applied by researchers and offered a means to study most, if not all, of the target species. This finding is central to the practical realisation of a unified strategy for non-volatile total flavour analysis as it potentially allowed the use of common HPLC instrumentation/hardware with selectivity for analyte group achieved by mobile phase selection. The only exception to this situation was the detection of lipids (due to their low adsorption in the UV range) where an alternative detection system would be necessary with the requirement for an additional switching valve in the column effluent

line.

The chromatographic procedure for all analyte classes is essentially the same and effectively involves partial resolution on one HPLC C_{18} RP analytical column followed by heart cutting onto a second, and photo diode array detection (Figure 3.4).

Figure 3.4 Stabilized Non Concentration Non Volatile Analysis (SNCNVA)



Switching is achieved by two computer controlled 6 port valves. To analyse all the different analytes, the method utilises a sequential multiple injection protocol, sampled from a single vial containing the chloroform/methanol liquor, and injected on to three different mobile phases depending on the nature and chemistries of the analyte/analyte group being targeted.

Selection of the mobile phases is a central step in the design and capability of the technique. If we consider the analyte groups and view their chemistries, we can establish three physicochemical categories based on the degree of ionization. Firstly the highly ionized low pKa acids, for example, ascorbic acid and dihydroxyfumaric acid; secondly analytes with intermediate pKa's including the flavonoids and phenolic acids, and lastly unionised species like the carotenoids and chlorophylls. To achieve good chromatographic performance in reversed phase HPLC, it is important to control the ionisation equilibria of ionised species to allow the molecular form to predominate within the analytical column. This is achieved by increasing the hydrogen ion concentration (ie. reduced pH) of the mobile phase. The extent of the reduction obviously depends on the equilibrium of the individual species, as reflected by their pKa values. In the above categorization, the low pKa group of analytes require a mobile phase approximately two pH units below their pKa values, at around pH 2.5. This pH is achieved using an aqueous phosphate buffer. The intermediate pKa group can be analysed using the higher pH system of 5%v/v aqueous formic acid. Although ionised species require pH control, other target analytes are very sensitive to acidic conditions and undergo degradation, for example, the chlorophyll and carotenoids. For these species, it is necessary to maintain the mobile phase near neutrality. Another important aspect in achieving chromatographic separation of a wide range of species is the mobile phase solvent strength required for elution from the column and gradient elution with binary and ternary solvent systems offers the required flexibility.

After initial development, three mobile phase systems were established. Ascorbic acid and low molecular weight/low pKa acids were analysed using a 50mM pH 2.5 phosphate buffer mobile phase under isocratic conditions. Phenolic acids and flavonoids were analysed using a binary gradient from 100% (5% aqueous formic acid) to 90% methanol:10% (5% aqueous formic acid) over 30 min. Finally, tocopherol, carotenoids and chlorophylls are analysed using a tertiary gradient from 80% methanol:20% water to 50% methanol:50% ethylacetate over 30 min. The analysis conditions selected for chlorophyll follow the conditions used by Yamauchi *et al.* (1985), in their investigation of the degradation products of the chlorophylls and carotenoids.

The final area for consideration is the chloroform/methanol solvent extraction system of the sample. As discussed earlier (Section 3.1.1), the solvent has a finite capacity for water

which, if exceeded, causes the system to separate into two immiscible phases comprising methanol/H_2O and chloroform. Although it would seem feasible to inject a 50µl volume of the liquor into a methanol mobile phase, there was concern about the viability of injecting into an aqueous mobile phase. Interestingly and somewhat unexpectedly few problems were experienced although injection volumes for ascorbic acid analysis had to be reduced from 50µl to 10µl in order to achieve good peak shape.

The final component of the SNCNVA technique is the removal of interfering non-volatile species. This is achieved by selectively 'heartcutting' the component(s) of interest from one analytical column to another and subsequent rinsing of the first column with the strong solvent, chloroform/methanol/H_2O.

The following functional description of the SNCNVA technique summarises the protocol employed. The starting mobile phase is directed through both columns arranged in series. After conditioning, the second main analytical column is switched out of the analysis tract allowing mobile phase to pass through the first pre analytical column and exit to vent. The sample is injected and, after a prescribed time, effluent from column 1 is directed to the main analytical column for a set period before the pre column is switched out of the analysis tract. The selected mobile phase gradient continues to elute material from the second analytical column until separation is complete. Finally the unwanted materials residing on the pre column after the selective cut are washed to vent using a chloroform:methanol:water mix. This whole multiple analyte/multi sample sequence including column conditioning and pre-column washing is automated under software control using the Shimadzu LC10 system.

Additional development work was performed to establish a protocol for the analysis of dehydro ascorbic acid to complement the analysis of ascorbic acid. The sequence involved derivatization with o-phenyl diamine within the chloroform/methanol liquor and subsequent HPLC analysis. Although the procedure appeared viable, it was not included in the standard SNCNVA sequence, serving more as a feasibility study.

An alternative approach to ascorbic acid analysis is C_{18} RP ion pair chromatography which potentially allows greater interaction with the stationary phase and thus larger capacity factors, a slight limitation with the system described above. The exact mechanisms of ion-

pair chromatography continue to be discussed, but, in practical terms an ion-pair agent is added to the mobile phase which associates or pairs with the analyte to allow good chromatographic separation of ionised species even at neutral pH's.

3.2 Experimental

3.2.1 Extraction and Solubilization

Certain of the procedures reported in this section result from developmental work described later in this section, however they are recorded here in a logical, rather than a chronological sequence. Cultivation of parsley and the harvesting protocol are described in Chapter 2, Materials and Methods.

3.2.1.1 Development of an Extraction Protocol

The following extraction variables were investigated to establish a practically viable procedure which maximised the degree of cell disruption, allowed minimum handling of the sample, and considered the design criteria established in Sections 3.0-3.1. Performance was monitored using photomicroscopy on the final extract to establish the degree of cell disruption.

- solvent system.
- vial geometry.
- type of homogenizer head.
- position of liquid surface relative to shaft.
- order of addition of solvent.
- duration of ultrasonication

3.2.1.2 Stability of Analytes in Chloroform/Methanol/Water Solvent

(i) Volatiles

SNCVA II analysis was performed on three separate samples of frozen parsley (1.5 g; 1993 harvest) stored at -65° C. Alpha tocopherol was omitted from the extracting solvent in two of the samples and stored in clear glass and amber glass/foil wrapped vials

respectively, under ambient/laboratory light conditions. The third sample was extracted, with the addition of α-tocopherol to the methanol aliquot (0.4% w/v) and transferred to an amber/foil wrapped vial. Samples were held under continuous ambient/laboratory light conditions and repeatedly analysed over a 48 hours period. The effect of extract storage time on menthatriene level is shown in Figure 3.5.

(ii) Non Volatiles

SNCNVA analysis was performed on two separate samples of frozen parsley (1.5 g; Sample 1 1994 harvest; Sample 2 1992 harvest) stored at -65°C. After analysis, the resultant solvent extracts were held at 20°C for 10 hours and re-injected. The first sample (Sample 1) was analysed for all three analyte groups. The second sample (Sample 2) was analysed for chlorophyll and carotenoids only (Table 3.1).

3.2.1.3 Extraction Criteria Test

SNCVA I analysis was performed on frozen parsley (1.5 g; 1992 harvest) stored at -65°C. After sampling the extract for SNCVA/GLC analysis, the remaining extract was decanted off leaving a compressed particulate plug in the bottom of the extraction vial. The vial was weighed to determine the mass of the particulate plug and 37.697g fresh chloroform : methanol solvent was added. The vial was sealed, shaken and allowed to stand for 10 minutes prior to repeat centrifugation (3,000 rpm) and SNCVA/GLC analysis. The level of fresh solvent added was determined from preliminary experiments which recorded the weight of the particulate plug before and after drying. These data allowed an estimate of the interstitial solvent within the residue plug and in turn the amount of solvent required to dilute this by a factor of ten (37.697g). Figure 3.6 compares the two SNCVA chromatograms with the scaling adjusted to reflect the dilution factor employed (ie x10).

3.2.2 SNCVA development and characterisation

3.2.2.1 Assembly and Set Up of the SNCVA I system

Instrumentation:
- Fisons Instruments 5300 Mega Gas Chromatography with manual Grob on column injector and FID detector (EL-580 amplifier).
- Fisons Instruments Minichrom data acquisition computer.

Components:
- VICI-AG Valco 2 position 4 port switching valve Ref No A4N4WE specification: 1/32"od, 0.25mm id, 4" standoff, manual switching.
- VICI-AG Valco 2 position 6 port switching valve Ref No A4N6WE specification: 1/32"od, 0.25mm id, 4" standoff, manual switching.
- Valve heating rope Electrotherm Ltd HT8 silicon rubber insulation.

Columns:
- 25m x 0.32mm id x 0.4 μm Chrompack CP-Sil 5 GLC column.
- 1m pre analytical column, specification as above.
- 0.5m x 0.32mm PEG deactivated fused silica retention gap.

Carrier Gas Flow:
- Grob injector, pressure controlled to yield 15 cm/s gas velocity
- Analytical column-separation flow, mass controlled to 15 cm/s
- Pre column-separation flow, mass controlled to 15 cm/s

Sample Handling: - sample volume 1μl (SGE 5μl), rinse volume 8μl (SGE 10μl).

Amplifier Sensitivity - 2^4 (attenuation)

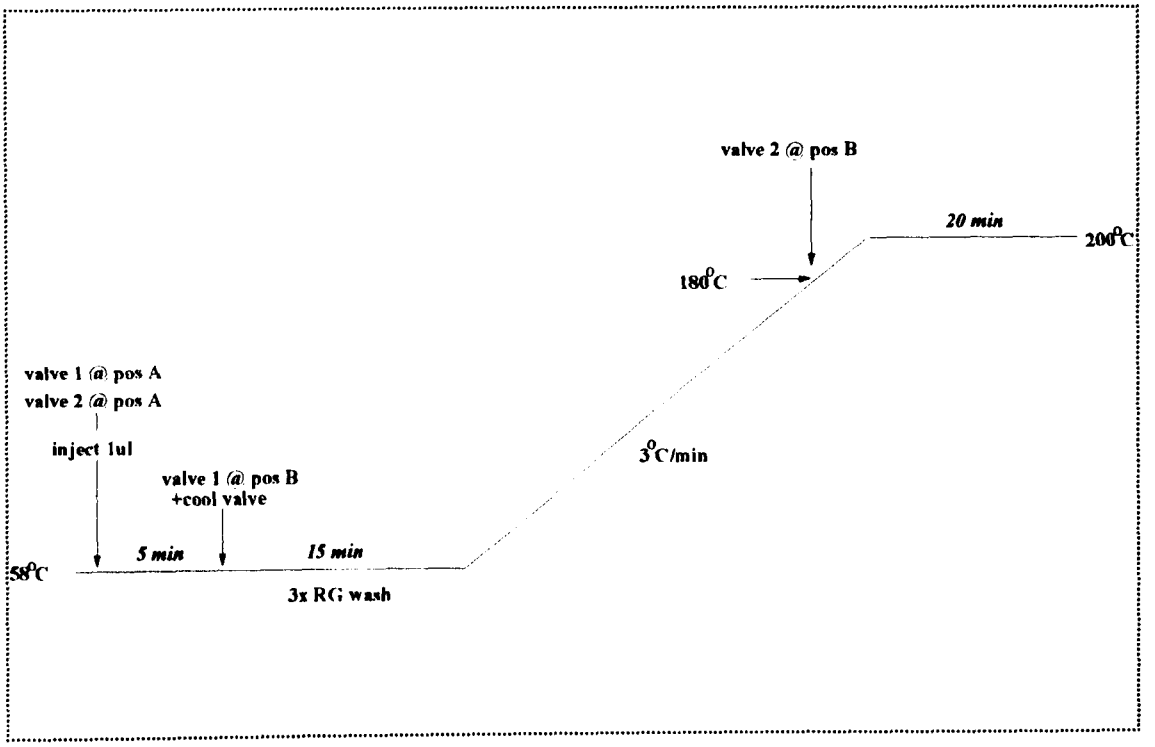
Data Acquisition - 0 - 1000 mV range, (ie Full scale 1000 mV = 16 x 10^{-12} A)

Peak Quantitation - see Section 3.3.2.1

Configuration: - see Figure 3.2

Oven Programme and Valve Timings:

SNCVA I GLC Oven Profile and Valve Timings.



3.2.2.2 Chromatographic Path Dynamics Test (SNCVA I)

'Methane test'

The system was configured with valve 1 and valve 2 at positions A (Figure 3.2) and methane (1 µl) injected.

Oven Condition: 30°C isothermal

3.2.2.3 Selection of Switching Valve Temperature (SNCVA I)

'Test Mix Analysis'

The system was configured with valve 1 and valve 2 at positions A (Figure 3.2). Chrompack Test Mix #31 in chloroform/methanol (2:1; 1µl) was injected at three separate valve test temperatures: (a) 60°C, (Figure 3.7), (b) 100°C, (c) 120°C. The system was reconfigured replacing the switching valves with 'Press Fit' glass column connectors. Chrompack Test Mix #31 (1µl) was reinjected, (Figure 3.8).

FID Attenuation: 64 (2^6)

Oven Condition: 60°C (5 min) to 120°C (15 min) at 5°C/min

3.2.2.4 Analysis of Parsley Leaf Oil using SNCVA I

The system was configured with valve 1 and valve 2 at positions A, (Figure 3.2). Parsley leaf oil (0.0086g) in 20 ml chloroform/methanol (2:1; 1µl) was injected, (Figure 3.9).

Valve Temperature: 100°C
FID Attenuation: 4 (2^2)
Oven Condition: 60°C (5min) to 120°C (5min at 3°C/min) to 200°C (20min at 10°C/min)
Parsley Herb Oil: produced by steam distillation

3.2.2.5 Comparison between SNCVA I and Steam Distillation for Sweet Marjoram

SNCVA I analysis was performed on frozen sweet marjoram leaves (1.5g) stored at -65°C. A sample (50g) from the same batch was analysed by steam distillation extraction (see Chapter 2 'Materials and Method') and the chromatographic data compared in Figure 3.10.

3.2.2.6 Comparison between SNCVA I and Steam Distillation for Parsley

SNCVA I analysis was performed on frozen parsley leaves (1.5g; 1992 harvest) stored at -65°C. A sample (50g) from the same batch was analysed by steam distillation extraction (Chapter 2.1.4) and the chromatographic data compared in Figure 3.11.

3.2.2.7 Effect of Cultivation Year and Location on the Volatile Composition of Frozen Parsley

SNCVA I analysis was performed on two separate samples of frozen parsley (1.5 g) stored at -65°C for 1 year (1992 harvest, green house grown) and 1 hour (1993 harvest, field grown) respectively (Figure 3.12).

3.2.2.8 Assembly and Set Up of the SNCVA II system

Instrumentation:
- Fisons Instruments 5300 Mega Gas Chromatography, automated Grob on column injector and FID detector (EL-580 amplifier).
- Fisons Instruments AS800 liquid injection autosampler.
- Fisons Instruments Minichrom data acquisition/control computer with I/O board.

Components:
- VICI-AG Valco 2 position 6 port switching valve Ref No A4N6WE specification: 1/32"od, 0.25mm id, 4" standoff, manual switching.
- VICI-AG Valco Digital Control Interface and valve pneumatics.

Columns:
- 25m x 0.32mm id x 0.4 µm Chrompack CP-Sil 5 GLC column.
- 0.1m x 0.32mm id methyl deactivated fused silica transfer tube.
- 0.8m x 0.32mm PEG deactivated fused silica retention gap.
- 0.05 x 0.53mm id methyl deactivated fused silica injector tube.
- 2 x 'Press Fit' 0.32mm glass tee.

Carrier Gas Flow:
- Grob injector, mass flow controlled at 0.6 ml/min
- Analytical column, pressure controlled to ~1 ml/min
- Tee arm flow (x2) ,mass controlled to 0.2 ml/min

Control System: - hardware, Appendix 3.1, software, Appendix 3.2.

Sample Handling: - sample volume 2µl, rinse volume 8µl (10µl SGE AS800).

Amplifier Sensitivity - 2^5 (attenuation)

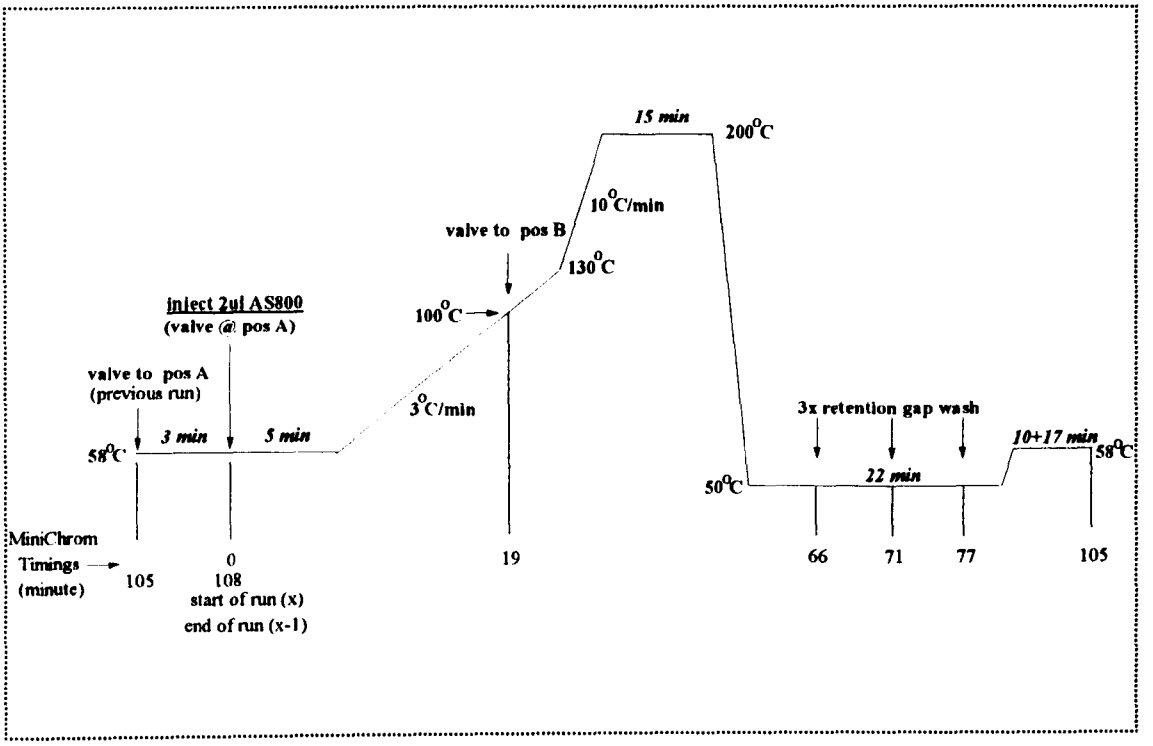
Data Acquisition - 0 - 1000 mV range, (ie Full scale 1000 mV = 32 x 10^{-12} A)

Peak Quantitation - see Section 3.3.2.5

Configuration: - see Figure 3.3

Oven Programme and Valve Timings:

SNCVA II GLC Oven Profile and Valve Timings.



3.2.2.9 Comparison between SNCVA I vs SNCVA II

SNCVA II analysis was performed on frozen parsley (1.5g; 1993 harvest) stored at - 65°C. This sample was compared to a sample from the same batch, run earlier using SNCVA I (Figure 3.13).

3.2.2.10 Evaluation of the Reproducibility of the SNCVA II Technique

SNCVA II analysis was performed on 24 separate samples of frozen parsley (1.5g; 1994 harvest) stored at -65°C. Chromatographic peak area were determined for the eight largest peaks and normalised for the weight of parsley analysed. Reproducibility statistics were performed on the data (Table 3.2; Figure 3.14).

3.2.2.11 Detector Calibration for Menthatriene

Menthatriene (0.012g) was dissolved in chloroform/methanol/water solvent (40g; 2 : 1 : 0.15). Aliquots of this solution (0.1, 0.2, 0.35, 0.5, 0.7g) were diluted with solvent (1.0g) and analysed (2µl injection) by SNCVA II. The complete sequence was performed in duplicate. Chromatographic peak area was determined and plotted against weight menthatriene injected (Figure 3.15).

3.2.2.12 Identification of GLC components

Parsley leaf oil (0.003g) in 30 ml chloroform methanol 2:1, (ex R.C.Treats Ltd Batch No 9C3295) was analysed by GC/MS. The sample (1 µl) was injected onto a 30m x 0.25mm poly dimethylsiloxane fused silica column (J&W DB1) coupled to a 'VG Trio' mass spectrometer using the standard SNCVA GLC oven profile and a mass scan range of 35-400 amu (Figure 3.16). The same parsley leaf oil sample was reanalysed using the chromatographic component of SNCVA II and compared with a standard SNCVA II analysis of frozen parsley (1.5 g; 1993 harvest) stored at -65°C (Figure 3.17).

3.2.3 SNCNVA development and characterization

3.2.3.1 Assembly and Set Up of SNCNVA system

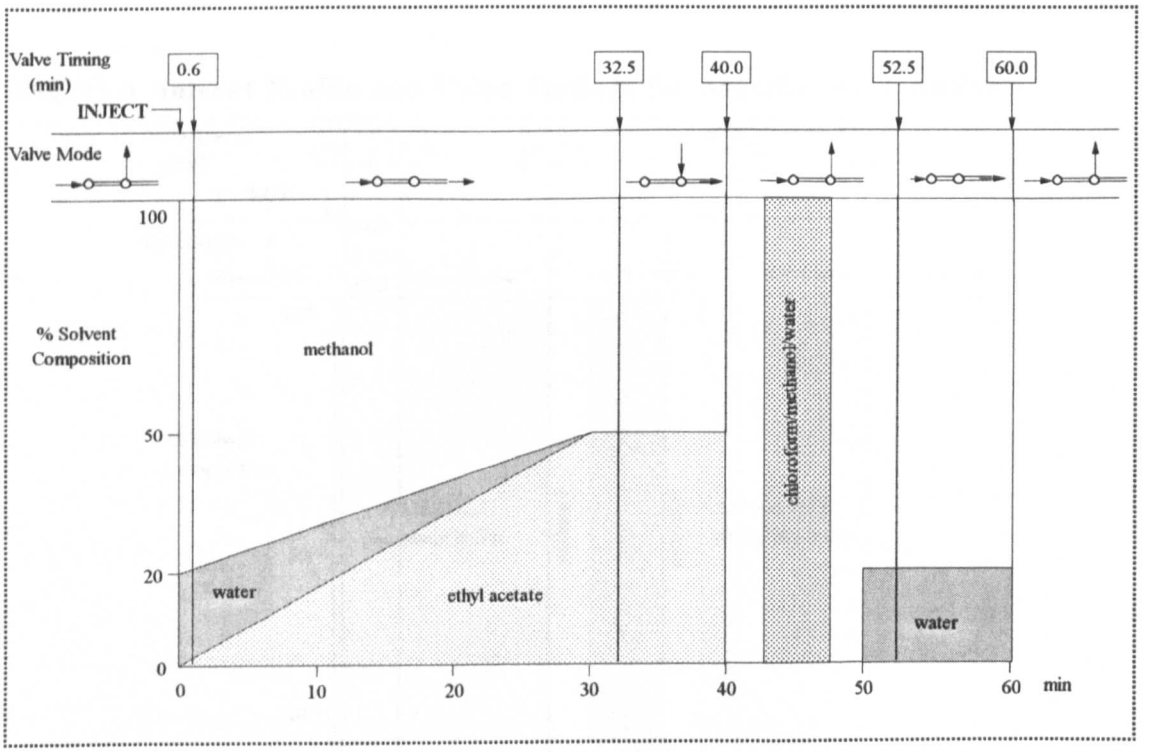
Instrumentation: Shimadzu High Performance Liquid Chromatograph consisting of
LC-10AD dual piston pump.
FCV-10AL 4 solvent low pressure gradient mixing valve.
SIL-10A autosampler with cooling option.
CTO-10A temperature controlled column oven.
SPD 6A Photodiode Array with D2 and W lamps.
FRC-10A Fraction collector with cooling option.
2 x 2 position 6 port valve with valve interface.
1 x 6 position solvent selection valve.
CBM-10A controller plus PC computer with LC10 software.

Columns: Beckman 4.6mm x 250mm Ultrasphere ODS 5µm LC column.
Beckman 4.6mm x 45mm Ultrasphere ODS 5µm LC pre-column.
System tubing: 0.01" x 1/16" stainless steel pre-cut tubing.

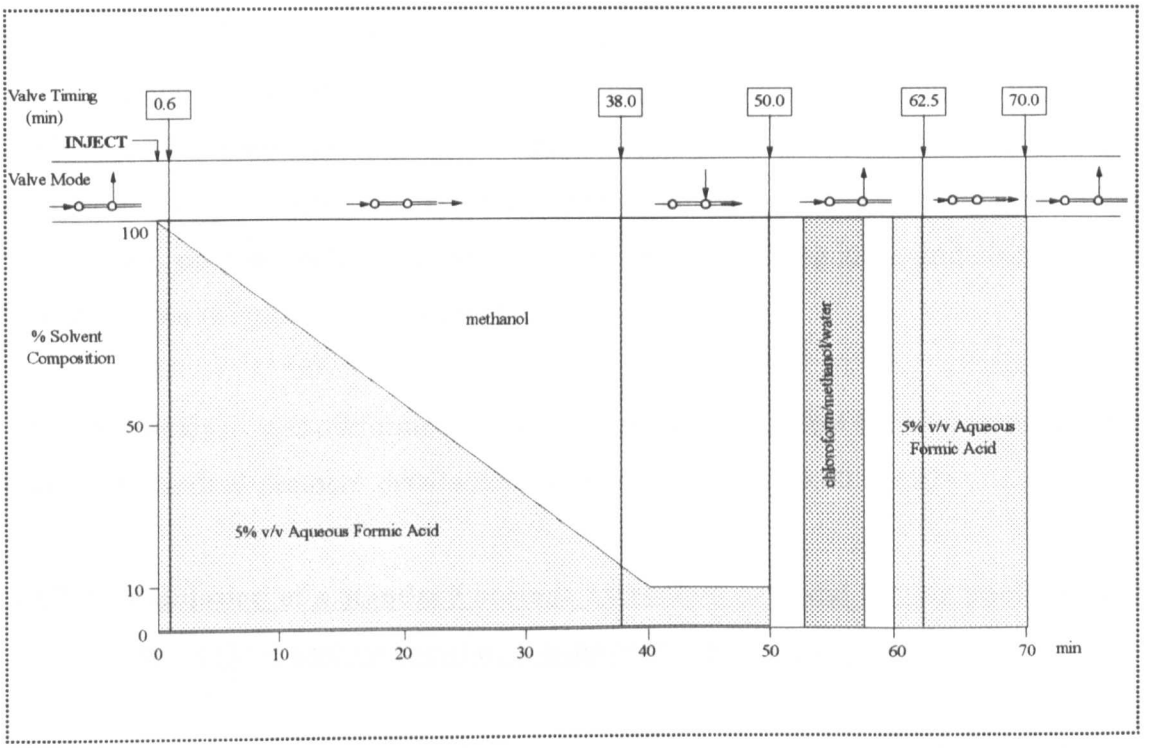
Solvents:	Non Aqueous	A. Methanol
		B. Ethyl Acetate
		C. Chloroform/Methanol/Water (2:1:0.15 by volume)
	Aqueous	D1 5% v/v Formic Acid
		D2 Grade A Milli Q water
		D3 50mM NaH_2PO_4/H_3PO_4 pH 2.5 buffer.
Solvent Flow:	1ml/min	
Sample Volume:	- chlorophyll run 50µl; flavonoid run 50µl; ascorbic acid run 10µl.	
Wavelength Range	- chlorophyll analysis:	260 - 550 nm
	- flavonoid analysis:	220 - 480 nm
	- ascorbic acid analysis:	200 - 350 nm
Data Acquisition	- Shimadzu LC10 System.	
Peak Quantitation	- see Section 3.3.3.1	
Control System:	- Shimadzu LC10 software.	

Gradient Conditions/Valve Timings

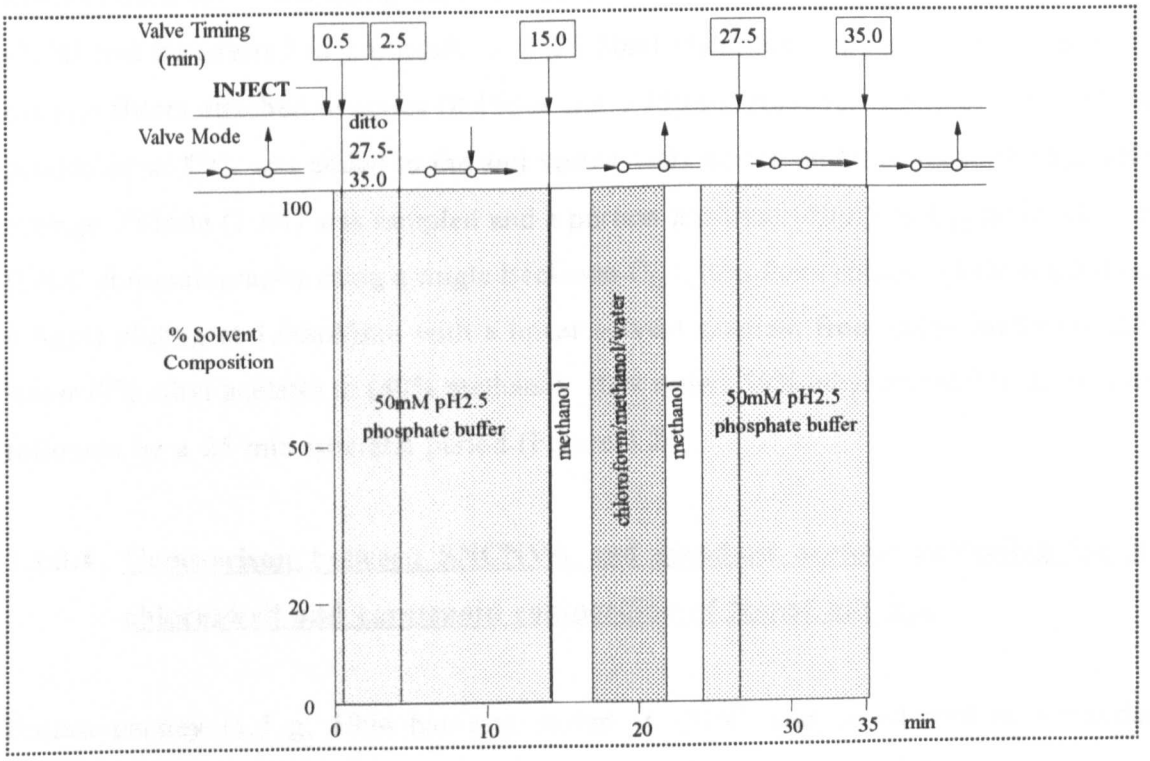
SNCNVA Solvent Profile and Valve Timings for Chlorophyll and Carotenoid Analysis.



SNCNVA Solvent Profile and Valve Timings for Flavonoid and Phenolic Analysis.



SNCNVA Solvent Profile and Valve Timings for Ascorbic Acid Analysis.



3.2.3.2 Chromatographic Characteristics and Column Switching Dynamics.

p-Coumaric acid (0.0033g), apigenin-7-glucoside (0.0043g) and apigenin (0.003g) were dissolved in 31.5ml chloroform/methanol/water (2:1:0.15), singly and in combination as a three component mixture. Portions of each solution (50µl) were analysed using a 50% methanol: 50% aqueous formic acid solution (5%v/v) under isocratic conditions with the analytical columns configured in series. Repeat injections of the 3 component mixture were made, progressively increasing the 'Valve 1' switching time and decreasing the 'Valve 2' time (Figure 3.19; Figure 3.4).

SNCNVA analysis was performed on frozen parsley (1.5 g; 1994 harvest) stored at -65°C, using the standard protocol detailed in Chapter 2/3 (Figure 3.20 - 3.22).

3.2.3.3 Validation of a standard acetone extraction protocol for the analysis of the chlorophyll and carotenoid composition of fresh spinach.

A single healthy spinach leaf, purchased from a local market, was washed, dried and cut into 1cm² squares and transferred (1.5g) to a standard SNCVA anti vortex vial containing 15ml 80% aqueous acetone cooled to ~ -70°C in acetone/solid CO_2 bath. The mixture was homogenised for 5 min using the standard SNCVA Polytron high shear cutting head at -70°C and transferred to a prechilled (5°C) 50ml Hamilton 'gas-tight' syringe with two syringe filters attached in series (0.45µm and 0.25µm). Aqueous acetone (25ml; 80 %), prechilled to 5°C, was added to the anti vortex vial, shaken and transfer to the Hamilton syringe. Filtrate (1 ml) was sampled and a portion analysed (50µl) by C_{18} reversed phase HPLC chromatography using a single Beckman C_{18} Ultrasphere column (4.6mm x 250mm x 5µm) eluting at 1.00ml/min with a linear solvent gradient from (80% methanol/ 20% water /0% ethyl acetate) to (40% methanol/ 10% water/ 50% ethyl acetate) in 20 minutes followed by a 25 min isocratic period (Figure 3.24).

3.2.3.4 Comparison between SNCNVA and standard acetone extraction for the chlorophyll and carotenoid composition of frozen parsley.

Frozen parsley (1.5 g; 1994 harvest), stored at -65°C, was transferred to a standard

SNCVA anti vortex vial and extracted with aqueous acetone, as described in the previous section for fresh spinach. Portions of the extract (50 ml) were analyzed using the full separation component of the SNCNVA method, see Section 3.2.3 (Figure 3.26).

SNCNVA was performed on frozen parsley (1.5 g; 1994 harvest) stored at -65°C (Figure 3.27).

3.2.3.5 Ion Pair Chromatography for the Analysis of Ascorbic Acid

A solution of tetra butyl ammonium phosphate solution (TBAP; 350 ml; 25 mM) was adjusted to pH 2.5 with the dropwise addition of 8.5% w/v phosphoric acid (5 ml in 350 ml TBAP ≈ 12.5 mM phosphoric acid). The solution was transferred into a 1 litre amber glass HPLC solvent reservoir, connected to solvent line 'B' and replacing the ethyl acetate solvent (Figure 3.4). Ascorbic acid (0.0098g) was dissolved in 31.5ml chloroform/methanol/water (2:1:0.15) and a 3ml sample transferred to a vial containing 1ml 12.5mM phosphate buffer (pH 2.5). The mixture was shaken and allowed to separate into two equal layers. A portion of the upper layer aqueous methanol layer containing the ascorbic acid was analysed using the SNCNVA system, eluting at 1.00 ml/min under isocratic conditions with a 10% TBAP (25 mM; pH 2.5) / 90% water, ion-pair-solvent (Figure 3.28).

3.3 Results and Discussions

3.3.1 Extraction and Solubilization

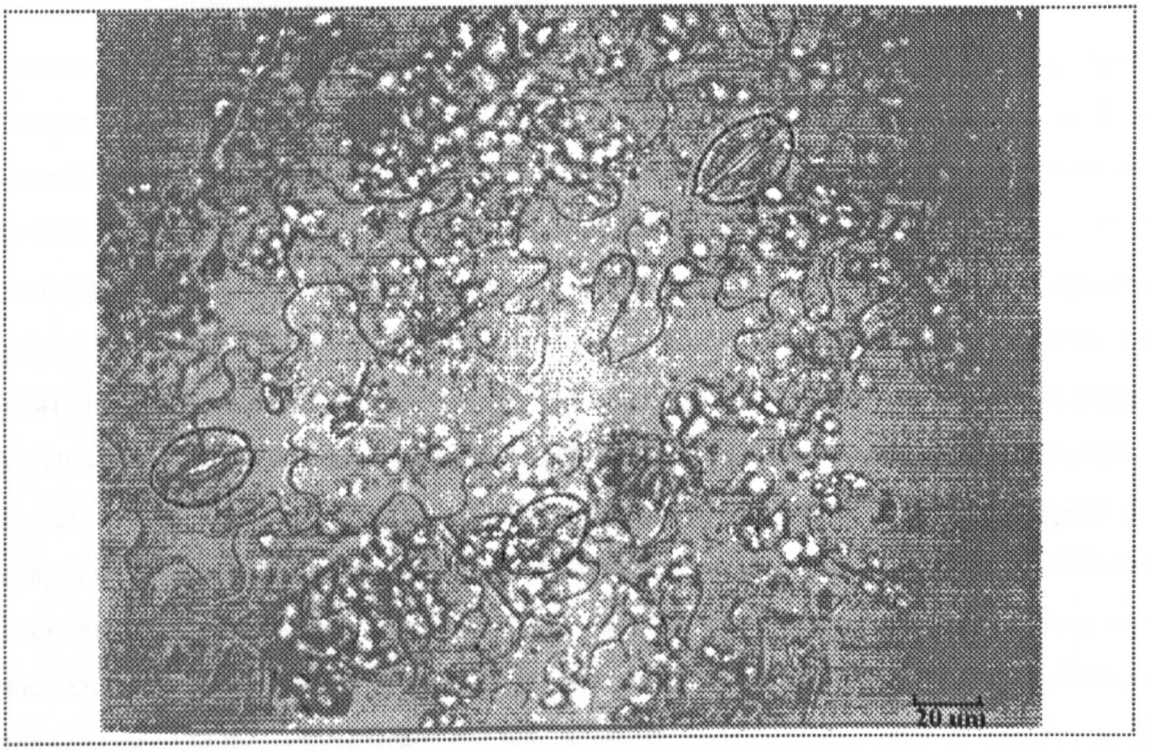
3.3.1.1 Development of an Extraction Protocol.

The solvent system used in this procedure consisted of 2 volumes chloroform to 1 volume methanol (Folch *et al.* 1957). The ratio of solvent to plant tissue was dependent on the water content of the tissue and dictated by the water solubilising capacity of the solvent system. A balance between maintaining a single phase solvent system, by not exceeding the water capacity, and maximising the amount of tissue and thus analyte concentration

must be arranged. Additionally, the amount of plant tissue required to obtain a representative sample must be taken into account as well as more practical constraints like the geometry and availability of vials and homogenizer heads. Preliminary development experiments established an arrangement whereby the homogeniser shaft entered the neck of the sample vial and was secured to the shaft with a cap and septa (Figure 3.1). This arrangement allowed the vial to be sealed during cell disruption and permitted the vial to be supported whilst immersed in a cryogenic ultrasonic bath. A typical sample vial had a nominal volume of 40ml and contained 30ml of the solvent system with 1.5 g - 2.0 g of frozen parsley leaves. The cultivation and freezing protocol for parsley is discussed in Chapter 2 (Materials and Methods).

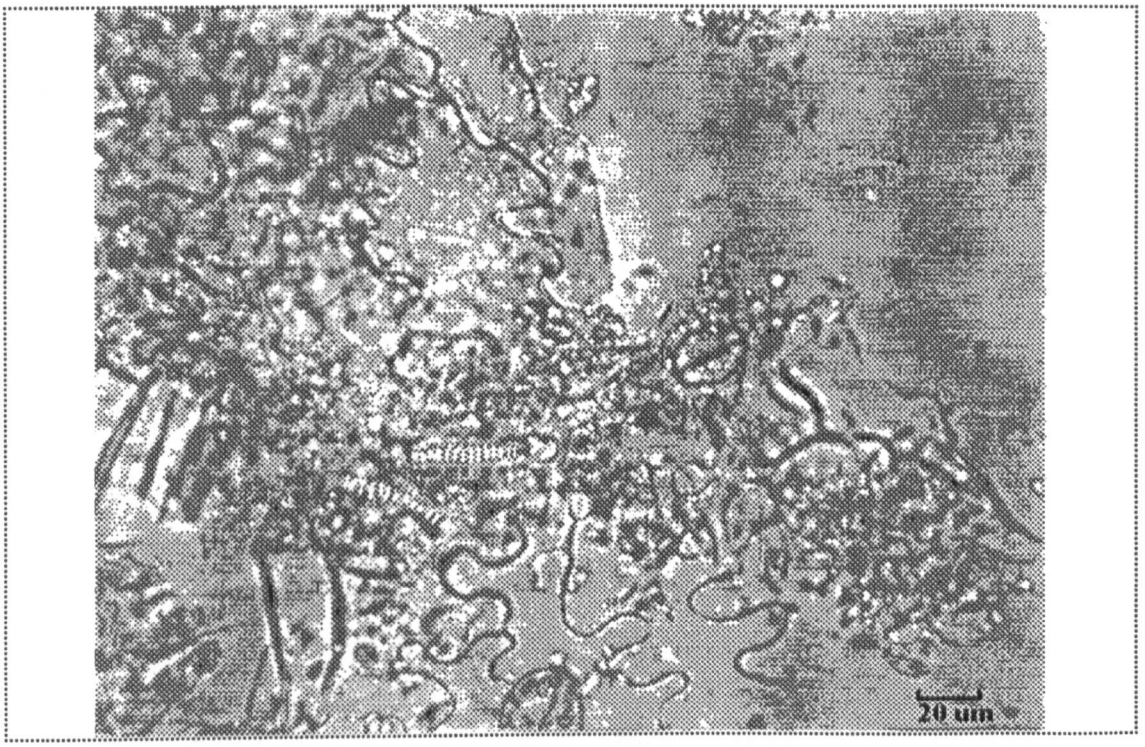
Initial experiments were conducted using a Ystral 9mm high shear homogenizer head and standard cylindrical vials. Under these conditions, only partial cell disruption was achieved, shown by the microscopy of the material (Plate 3.1).

Plate 3.1 Photomicrograph of Parsley Extract (Ystral Homogenizer)



The Ystral unit was replaced with a Polytron 12mm head with knives, designed to impart a cutting action rather than high shear compressive/expansive forces. Additionally the

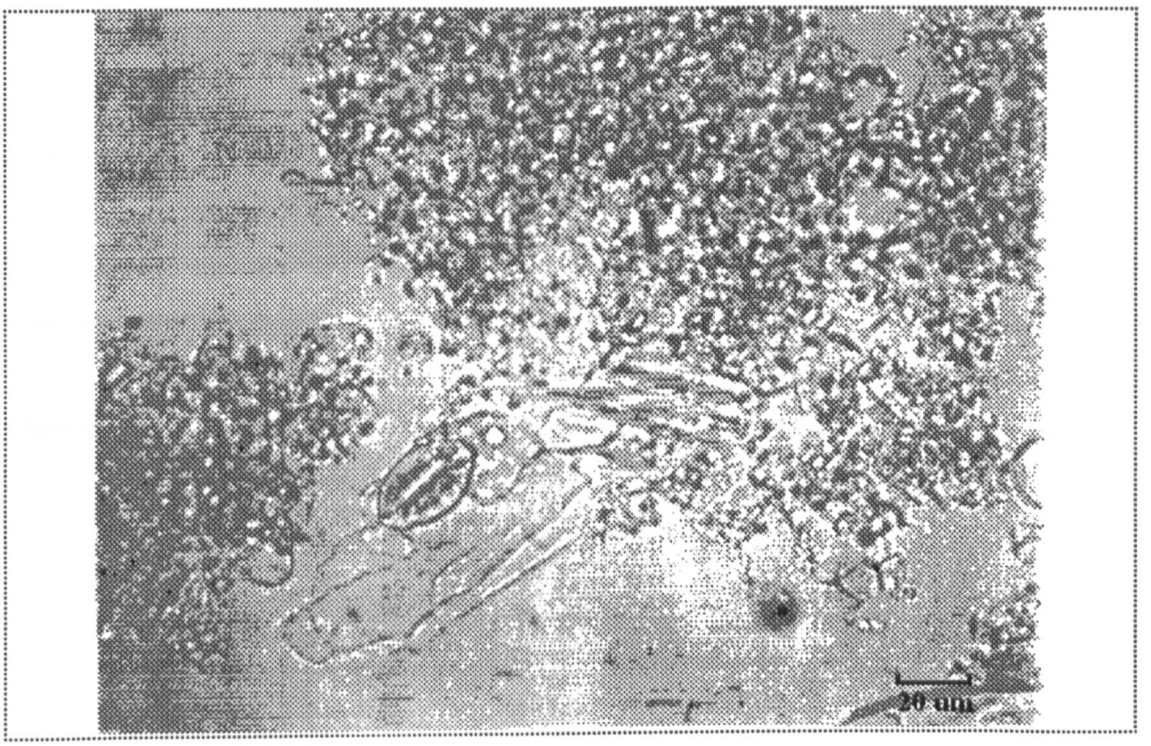
Plate 3.2 Photomicrograph of Parsley Extract (Polytron Homogenizer)



standard cylindrical vial was replaced with antivortex units, to permit higher homogenization speeds without sample 'bumping'/loss. These vials were designed and specifically manufactured for the work in this thesis. The units have a '4 clover leaf' profile in ambered glass with nominal volume of 40ml. They are designed to be centrifuged at moderate centrifugal force to permit the sedimentation and removal of particulate material within the extraction liquor. Under these conditions, extensive cell disruption was observed. However there still remained isolated regions where cell clusters existed (Plate 3.2). It was interesting to note that the most resilient cells appeared to be the epidermal cells. These cells are associated with the polymeric cuticle layer and this may be the reason for their resistance to solubilization/disruption. The final development sequence involved adding the extraction solvents as component parts, starting with methanol. This provided a means to produce a more concentrated homogenate to increase the contact with the cutting heads but also allowed the crystalline water to dissolve in the methanol antifreeze mix. A potential consequence of having a liquid system is that it is more sensitive to the compressive/expansive forces present during shearing. Under these conditions total cell disruption is achieved with the bulk of the particulate material

appearing as clusters of subcellular particles with only a very limited number of small cell clusters remaining (Plate 3.3). These sub cellular structures are about 2 μ - 5 μ in diameter and need to be considered further in terms of their potential to act as a flavour sink.

Plate 3.3 Photomicrograph of Parsley Extract (Polytron /Methanol)



From histology reference books, the primary structures present are likely to be (a) chloroplasts (2 μ - 5μ), (b) phloem/xylem fragments and (c) cell wall fragments. Chloroplasts are potentially of concern as they contain approximately 50% protein and 50% lipid and offer a significant sink for flavour. However their visual presence may result from the structural protein of the chloroplast, whilst the offending lipid component may in fact be solubilized by the powerful solvent system. Certainly chlorophyll, an intimate component of the chloroplast, appears to be effectively removed from the structure, based on the light brown coloration of the residue material. The other remaining structures are believed to consist of hydrophilic polymeric structures, namely xylem, phloem and cell wall fragments and should not have a capacity to accumulate flavour.

The final component of the extraction procedure involved centrifugation of the extraction liquor within the sample vial, to sediment particulate material. These conditions produced a single, dark green, visually clear extract layer with a compact pellet of light brown

sediment. Details of the extraction procedure are reported in Chapter 2 ('Materials and Methods').

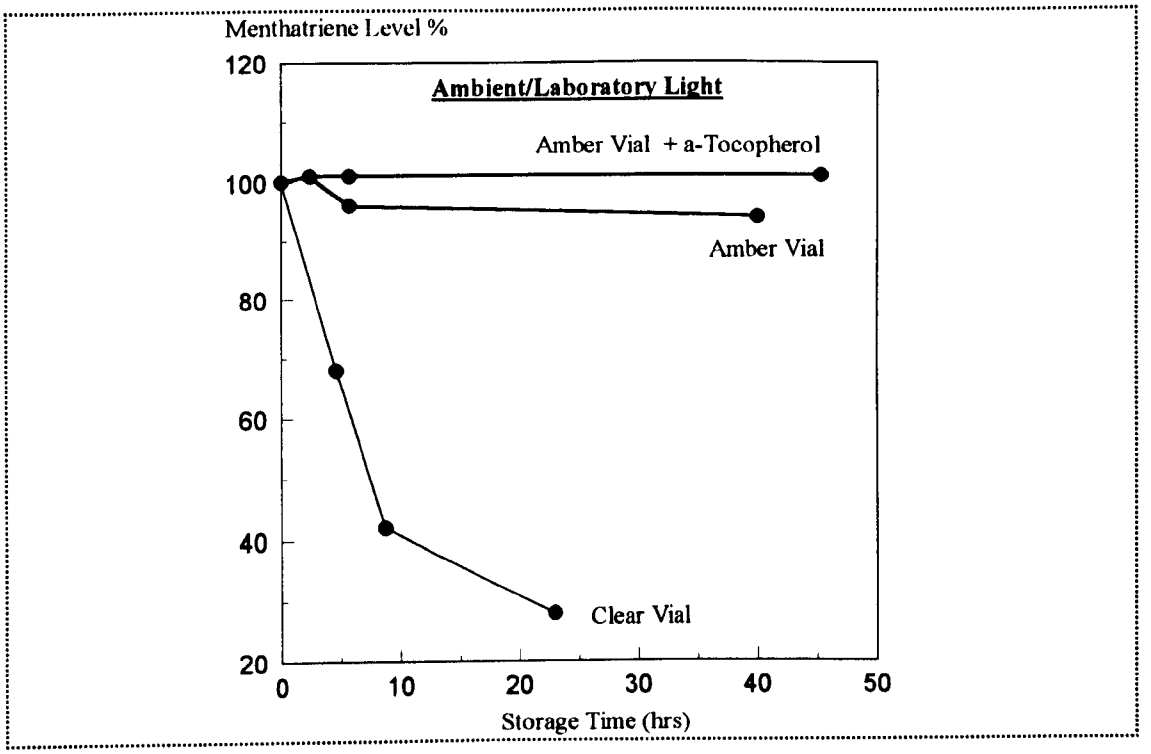
Experimentation to test the extraction efficiency of this procedure is reported below.

3.3.1.2 Stability of Analytes in Chloroform/Methanol/Water Solvent.

(i) Volatiles

Menthatriene showed rapid degradation when stored in chloroform/methanol/H_20 under ambient/laboratory light conditions (Figure 3.5).

Figure 3.5 Stability of Menthatriene in the SNCVA Extraction Solvent



Removal of light, with or without the addition of α-tocopherol extensively stabilised menthatriene over a period greater than 48 hours. Typical maximum holding times, prior to extracted analysis, do not exceed 5 hours, and therefore light exclusion offers an effective stabilisation protocol within the SNCVA method. Since menthatriene is a tri-unsaturated molecule and particularly prone to oxidation, this suggests that other volatile

species will also be stabilised by this approach. α-Tocopherol does not appear to offer any significant advantage to stabilisation, and its inclusion would compromise analysis of endogenous α-tocopherol using the SNCNVA method.

(ii) Non-Volatiles

From the data presented in Table 3.1, ascorbic acid and chlorophyll 'a' show significant levels of degradation although for chlorophyll the situation is inconsistent.

Table 3.1 Stability of Non Volatile Analytes in Chloroform/Methanol/Water

SNCNVA Analyte Window	Analyte	Peak Area			Percent Degradation After 10 Hours
		Wavelength	Time 1	Time 2	
Chlorophyll / Carotenoid	Sample 1 (1994 Harvest)				
	β-carotene	440 nm	157.5	152.3	3
	chlorophyll 'a'	430 nm	909.9	498.9	45
	chlorophyll 'b'	465 nm	400.8	362.7	9
	pheophytin 'a'	410 nm	0.0	600.9	-
	Sample 2 (1992 Harvest)				
	β-carotene	440 nm	230.5	230.2	< 1
	chlorophyll 'a'	430 nm	1408.3	1388.5	< 1
	chlorophyll 'b'	465 nm	728.7	741.1	- 2
	pheophytin 'a'	410 nm	21.7	52.9	-
Phenolic / Flavanoid	Sample 1 (1994 Harvest) (apigenin glucoside)	340 nm	1792.1	1768.8	1
	unknown phenolic	302 nm	406.1	415.7	-2
Ascorbic Acid	Sample 1 (1994 Harvest) ascorbic acid	245 nm	681.4	560.9	18

Chlorophyll degradation appears to be correlated to the formation of pheophytin and might be expected to occur under acid conditions. The SNCNVA protocol applied to the first sample (sample 1 in which chlorophyll degradation is observed) involved performing

ascorbic acid analysis, using phosphoric acid as the mobile phase, prior to the re-analysis of the extract. It is possible that carry over from the acidic conditions of the previous ascorbic acid analysis could be responsible for the observed change. Certainly in the absence of ascorbic acid analysis, as was the case for 'sample 2', no significant degradation of chlorophyll was observed. The data generated in this section were obtained at an early stage of SNCNVA development. Subsequent development optimised the order of analysis allowing the more labile compounds to be analysed first, as well as optimising the conditioning sequence between analyte windows. Over an extended number of analyses within this thesis, no further evidence of chlorophyll degradation and pheophytin formation was observed in the context of stability.

3.3.1.3 Extraction Performance.

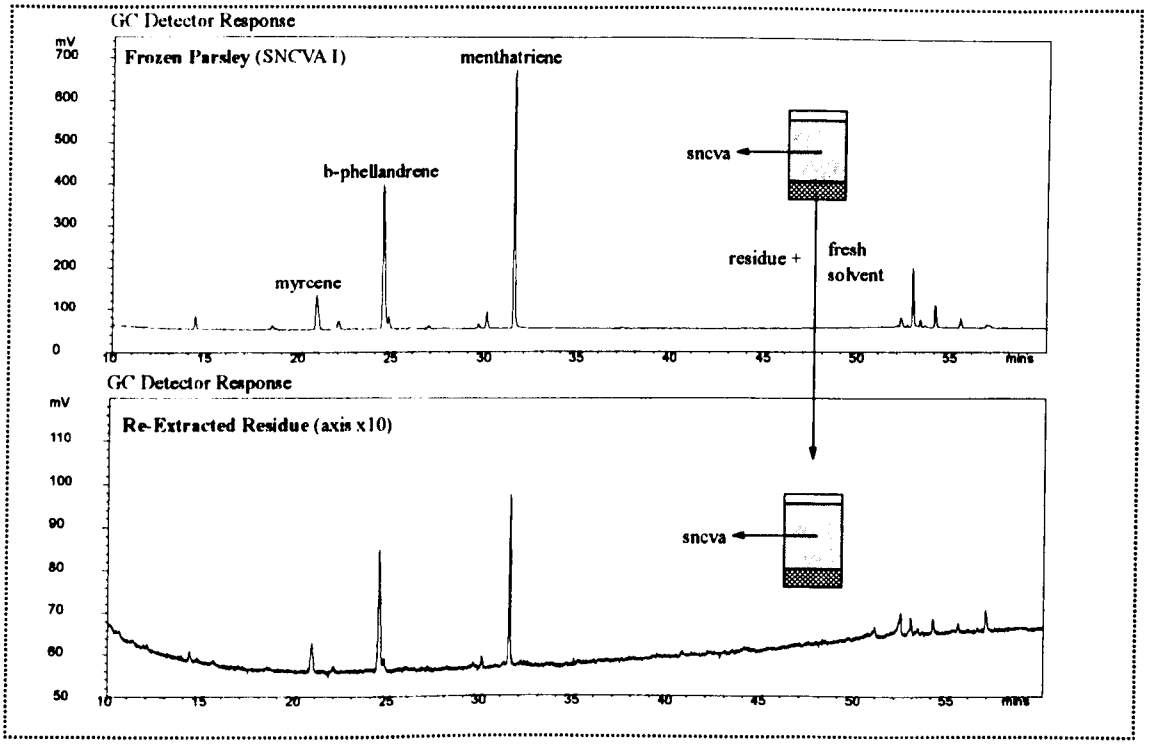
By comparing the chromatogram for the original frozen parsley extract with the re-extracted residue (Figure 3.6), it is visually apparent that the re-extracted chromatogram, representing a 10 fold dilution/10 fold scaling, is similar in peak magnitude terms, and more importantly the ratio of the peaks remain approximately constant. The important feature of this result is that dilution of the interstitial solvent is mirrored by flavour component concentration. This suggests the absence of any localised concentration of flavour within the particulate residue, capable of redistributing into the fresh solvent. The tentative conclusion from these data is that all cellular structures capable of acting as flavour sinks are solubilised. Formation of a solution is an important component in achieving quantitation within this technique. It should however be noted that volatile flavour only represents a limited physicochemical class of chemicals, typified by low MW and lipophilic character. Higher molecular weight polar and non polar species represented by our 'target analysis' in Table 1.4, also need to be considered.

'Extraction Summary'

For parsley, chloroform/methanol (2:1) was a suitable solvent system, which coupled with stabilizing conditions, prevented artifactual change as the analytes were removed from their natural cellular stabilisation mechanisms. Methanol was used to inhibit enzyme activity; light was excluded to prevent photochemical reactions and solubilisation temperatures were held at -70°C in a closed system to minimise chemical reactions and

evaporative loss. The sequence involved transferring frozen parsley at -60°C into a precooled specially designed amber glass antivortex vial. Aliquots of methanol and chloroform were added, in that order, and the sample sheared using a Polytron cutting shaft at 30,000 rpm and -60°C with ultrasonication between additions. The sample vial, sealed at all times to minimise evaporative loss, was transferred to a bench centrifuge to remove particulate material. The clear green liquor was transferred and sealed into GLC/HPLC amber autosampler vials for chromatographic separation. As part of the development sequence, α-tocopherol (0.4% w/v) was initially added to methanol and used as the standard extraction protocol for the SNCVA I technique. Subsequent development established this antioxidant could be omitted and thus was implemented in the extraction protocol for SNCVA II/SNCNVA (see 'Material and Methods', Chapter 2).

Figure 3.6 Effect of Residue Re-Extraction on Volatile Composition.



3.3.2 SNCVA development and characterisation

3.3.2.1 Assembly and Set Up of the SNCVA I system

In assembling the components of the SNCVA I system, particular attention was required in coupling the fused silica tubing to the valve ports to ensure minimum dead volumes.

The length of the retention gap was tailored to allow the flooded front of the solvent to approach the valve but not enter it. To control the temperature of the valves, platinum Pt-100Ω sensors were cemented to the body of the valves adjacent to the rotor and connected to the sensing/control circuitry of the gas chromatograph . Similarly the standard GC zone heaters associated with this circuitry were replaced with heating rope and wound around the rotor and standoff assembly of the valves. The heater zone temperature was set to the required value using the GC keyboard (zone 2; Figure 3.2).

'Component Quantitation'

The output voltage from the flame ionizing detector amplifier (FID) was acquired using a Fisons Instruments Minichrom system. The chromatographic data was integrated using the systems internal algorithm and assigned baselines manually checked and edited where appropriate. The area for each peak was normalized for the weight of frozen parsley analysed and expressed as the peak area (unattenuated) per gram frozen parsley (performed within Lotus 1-2-3 spreadsheet), using the following equation.

$$\text{Peak Area } (V.s.g^{-1}\text{frozen parsley}) = S \times V_c \times IPA \times \frac{(V^{meth} + V^{chl})}{V_{inj}} \times 1/W$$

where S = Amplifier Sensitivity (SNCVA I $=2^4$; SNCVA II $=2^5$).

V_c= Voltage Conversion Factor (μV to V = 10^{-6}).

IPA= Integrated Peak Area (μV.s)

V^{meth}= Volume of Methanol (10 ml)

V^{chl} Volume of Chloroform (20 ml)

V_{inj} = Volume of Extract Injected (SNCVA I=0.001ml, SNCVA II=0.002ml).

W = Weight of Frozen Parsley (g)

In this equation, no account is made for the volume of liquid, contributed by the parsley sample, to the total extract volume. The potential effect on the concentration of the sample extract (and thus the peak area), based on a 90% water content and comparing two samples at 1.00 g and 2.00 g (target weight=1.50g), would be of the order of 2-3%.

For comparative purposes (quantitative), chromatograms are displayed using a normalized 'GC detector response' axis to account for the weight of parsley analysed. To normalize a series of chromatograms, a single sample was selected as a reference, which displayed a large menthatriene peak from a large sample weight. Other samples are scaled to this chromatogram by assigning a new y-axis display scale based on the following equation:-

$$\text{y-axis scale (sample x)} = \text{y-axis scale (reference)} \times \frac{\text{Weight of sample x}}{\text{Weight reference sample}}$$

Scaled chromatograms are subsequently overlayed for comparison.

For steam distillation samples a similar comparison protocol was used. The weight of parsley used in the calculation was estimated from the proportion of solvent extract diluted (0.5g; total 15g) into the chloroform/methanol injection solvent (30 ml) and the weight of parsley analysed (50g; Section 2.14). This calculation assumes that all the volatiles in the parsley sample are transferred to the extracting solvent during steam distillation.

3.3.2.2 Optimisation of Conditions for SNCVA I Analysis.

'Methane Test'

The methane peak showed excellent peak symmetry indicating the absence of dead volumes within the chromatographic path (data not shown). This result was extremely welcome considering the many components within the system and the large number of connections. The methane test shows that the potential for good chromatography exists within this system.

'Selection of Switching Valve Temperature'

With the switching valves at 60°C, the test mix showed a rather disappointing chromatogram with the solvent peak showing an extended tail and the presence of a 'hump' in the centre of the chromatogram (Figure 3.7). Closer examination of the chromatogram shows reasonable chromatographic separation, in terms of both retention times and amounts. One explanation is that the system is prone to condensation. Removal of the valves and replacement with 'quick fit' connectors clearly identified the valves as

Figure 3.7 Test Mix 31 (Valves @ 60°C, FID Attenuation 64)

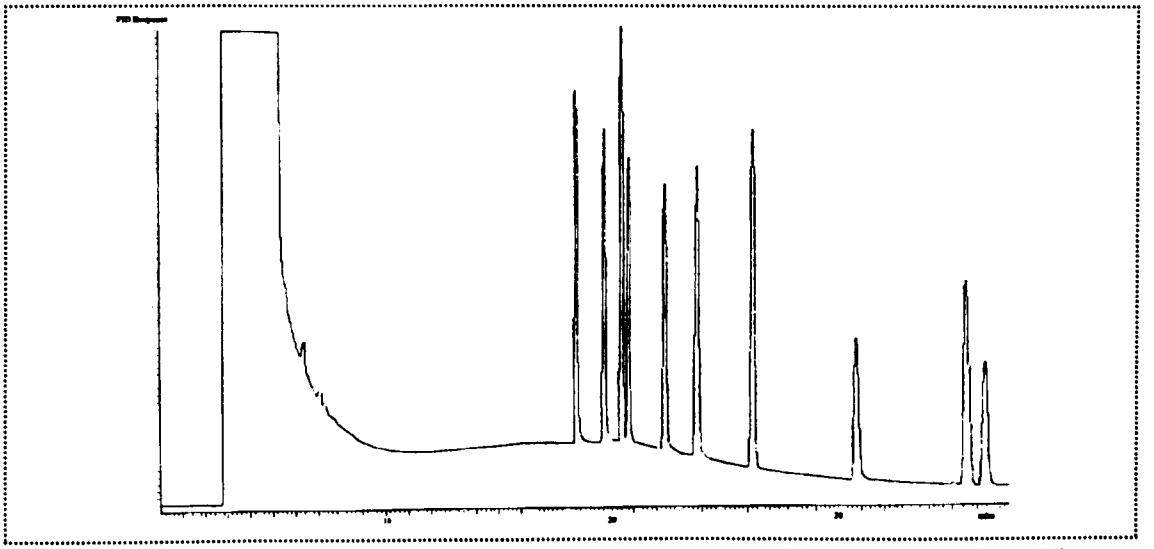


Figure 3.8 Test Mix 31 (PressFit Glass Connectors, FID Attenuation 64)

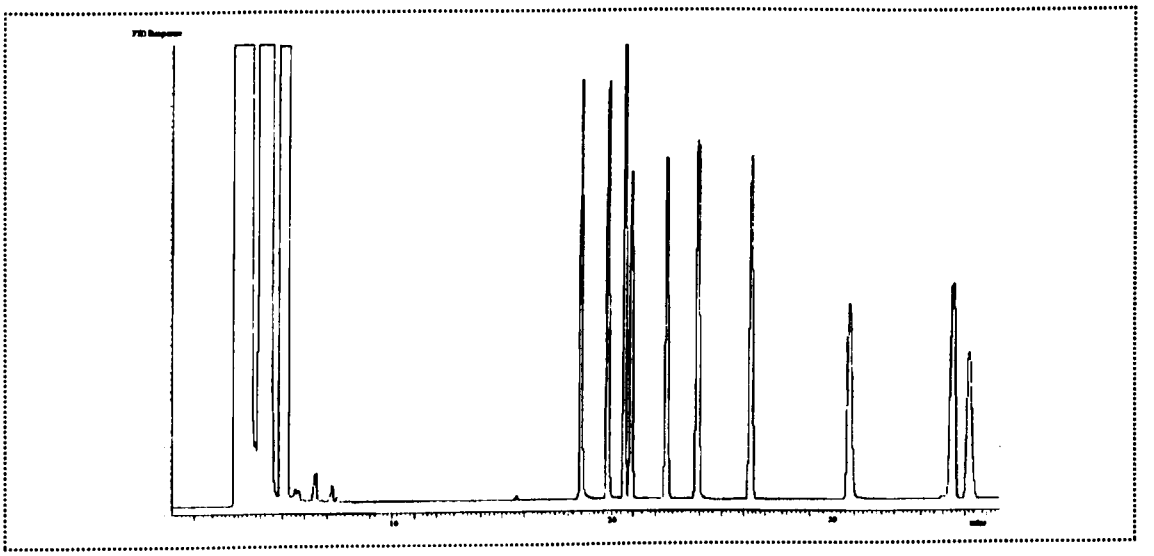
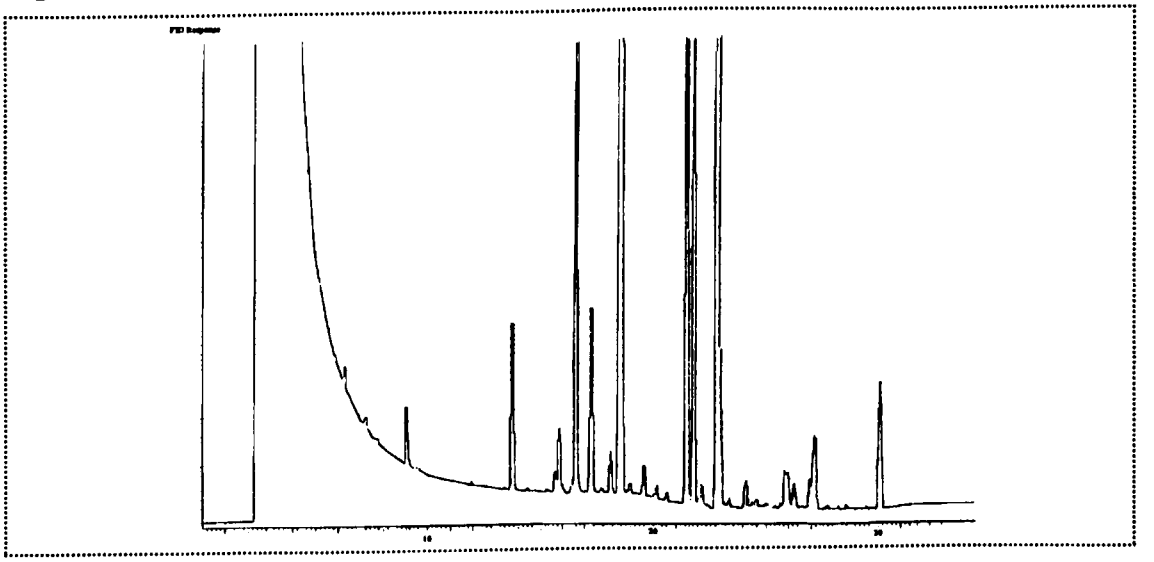


Figure 3.9 Parsley Leaf Oil (Valves @ 100°C, FID Attenuation 4)



the source of the problem (Figure 3.8). This chromatogram showed excellent chromatography of the test mix/solvent system and established the viability of direct on-column injection of the chloroform/methanol solvent. The solvent problems observed with the valves in position is likely to be due, partly to condensation and partly to selective partitioning of the solvent into the rotor material of the valve. This material is a mixture of PTFE and PEEK and is selected for inertness, temperature tolerance and reproducibility of switching and few alternative polymers exist. It is the chemical nature of the polymer which gives rise to the partial incompatibility to chloroform and indeed other chlorinated solvents, especially dichloromethane. The options as far as SNCVA are concerned are:- (a) redesign the solvent system (b) redesign the chromatographic path to exclude the valves and (c) heat the valves to establish the ratio of effects between condensation and partitioning. As part of the third option, heating the valves to 100°C significantly reduced the magnitude of the solvent phenomenon (see following section). Valve temperatures above 100°C did not improve the situation further.

'Analysis of Parsley Leaf Oil using SNCVA I'

To establish if parsley tissue can potentially be quantified by the SNCVA method, parsley leaf oil dissolved in chloroform/methanol at levels calculated to reflect tissue extract concentrations was analysed. With the switching valves set to the optimum temperature (100°C) and the sensitivity of the FID amplifier increased, the resultant chromatogram established the workability of the system (Figure 3.9).

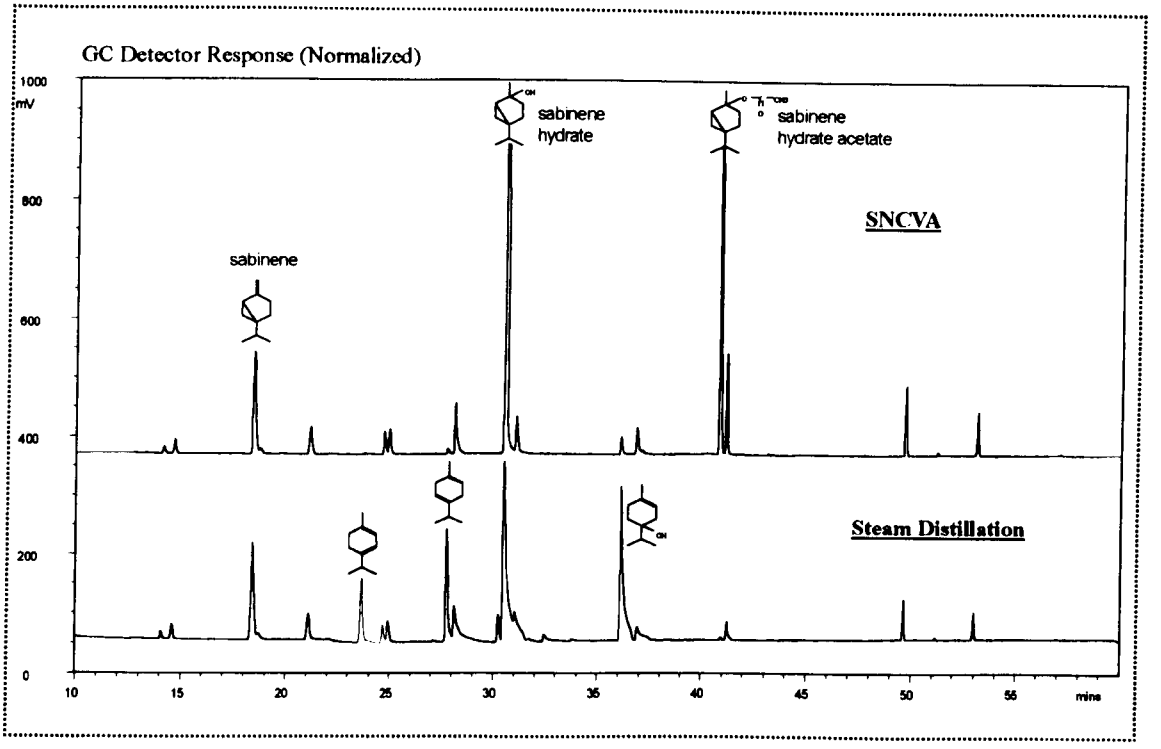
3.3.2.3 Comparison between SNCVA I and Steam Distillation Extraction.

'Sweet Marjoram'

From the chromatograms shown in Figure 3.10 it was established that steam distillation produced a very different volatile profile to SNCVA. If the SNCVA chromatogram is compared with the complex liquid nitrogen trituration analysis of sweet marjoram performed by Fischer *et al.* (1987), excellent agreement is seen. Equally if we compare our steam distillation chromatogram with Fischer's comparable steam distillation analysis similar changes are observed. These changes have been shown to be based on the ring opening and hydration of the sabinene moiety (Fischer *et al.* 1987, 1988; Chapter 1.61). The significant feature of these results, and the basis of the experiment, was to establish

whether the stabilisation strategy used in the SNCVA technique was effective in assaying the reactive environment of sweet marjoram. The data presented clearly established the ability of the SNCVA method to work effectively with labile systems and was a key component in the approach and objectives of this study. Application of this method to other plant tissue systems should allow the generation of novel data and form the basis to extend our current understanding.

Figure 3.10 Effect of Extraction Method on the Volatile Composition of Sweet Marjoram

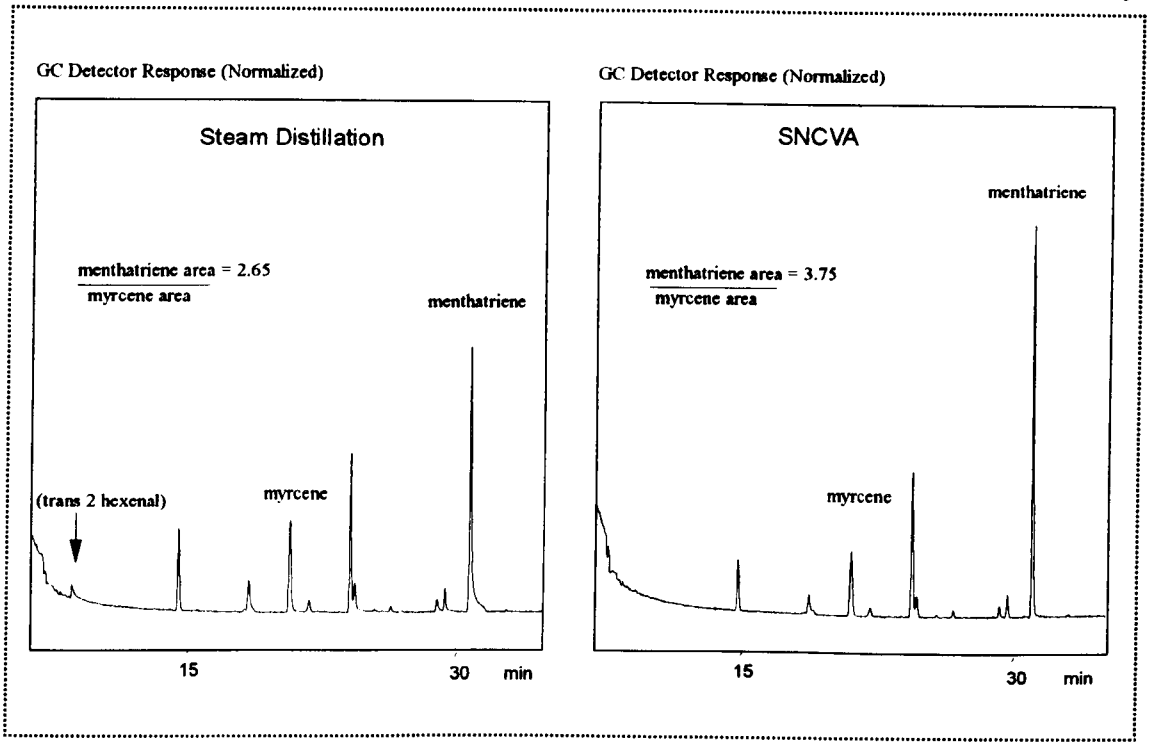


'Parsley'

From the chromatograms shown in Figure 3.11 it is clear that the extensive change exhibited by sweet marjoram are not reflected in parsley. The chromatogram from steam distilled parsley and SNCVA show many similarities in the type and amounts of compounds present. However, the tentative appearance of trans-2-hexenal in the steam distilled sample and the relative size of the menthatriene peak compared to the other peaks of the chromatogram show that some differences do exist. In this comparison, myrcene has been selected from storage stability data (Figure 4.2/Chapter 4), as a relatively stable monoterpene and used to express the relative amounts of menthatriene present in each analysis. The ratio of menthatriene to myrcene peak areas for steam distillation is 2.65

compared to 3.75 for SNCVA and tentatively supports the improved retention of the labile menthatriene molecule within SNCVA.

Figure 3.11 Effect of Extraction Method on the Volatile Composition of Parsley.

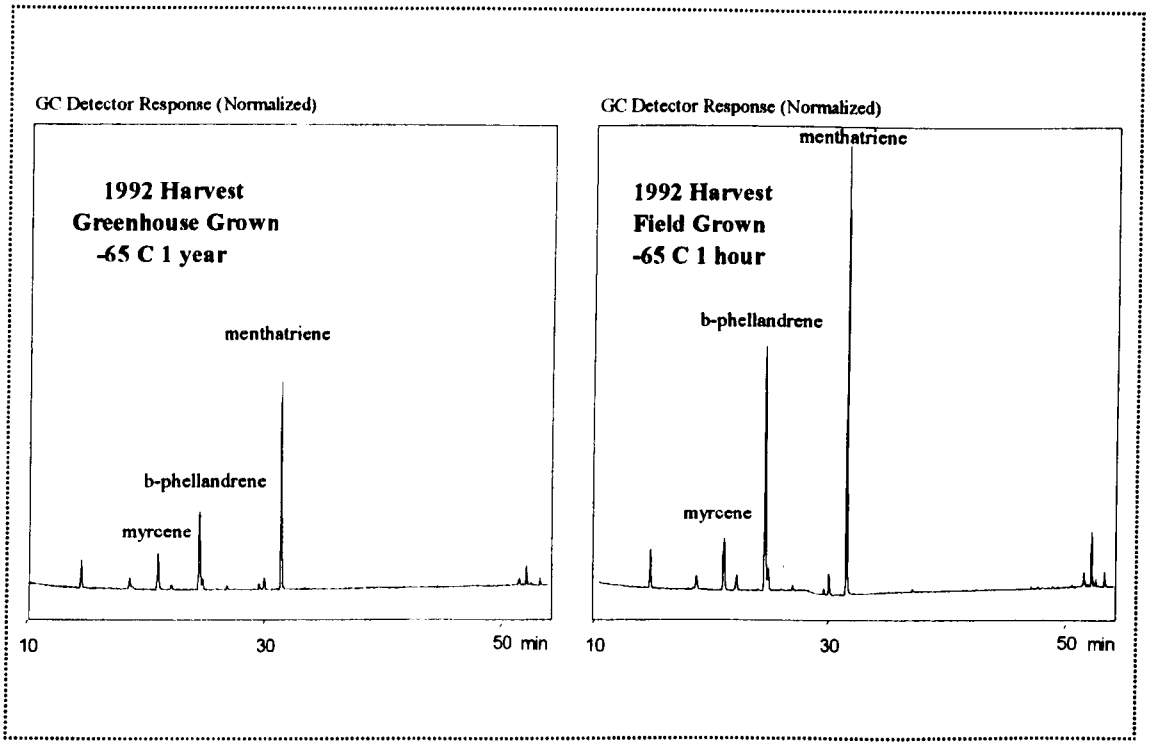


3.3.2.4 Effect of Cultivation Year and Location on the Volatile Composition of Frozen Parsley.

In a study of this type, involving the analysis of endogenous species as a function of post harvest processing, it is important to establish analytical protocols which are sufficiently rapid for 'real time' analysis or develop protocols which hold the material so that it can be analysed at a later date. From the review in Chapter 1, Philippon *et al.* (1986) and Duden *et al.* (1981,1982) identified the lability of total aroma chemicals and polar plant lipids at typical domestic freezer temperatures. These authors also showed that the degradation phenomena can be arrested at temperatures of -50°C to -65°C. As part of the protocols used in this study, -65°C frozen storage was used to arrest change and has been experimentally confirmed, over an extended number of analyses, within this study (data not shown). To establish the variation between cultivation year and location, one year old parsley (1992 harvest, green house grown) stored at -65°C was compared to 1 hour old

parsley (1993 harvest field grown) stored under similar conditions (Figure 3.12). From the data, a significant difference in the level of compounds present was observed (menthatriene level, 1992 = 274 $\mu g.g^{-1}$, 1993 = 488 $\mu g.g^{-1}$,see Sections 3.3.2.8/3.3.2.5), however very similar profiles were displayed (Figure 3.12). In comparing , 1993 field grown harvest with 1994 field grown harvest (menthatriene level, 1994 = 464.8 $\mu g.g^{-1}$, see Section 3.3.2.7), both similar compound level and profiles were shown, suggesting the low levels in the 1992 harvest was due to green house cultivation.

Figure 3.12 Effect of Cultivation Year and Location on the Volatile Composition of Frozen (-65°C) Parsley.



3.3.2.5 Assembly and Set Up of the SNCVA II system.

In assembling the components of the SNCVA II system (Figure 3.3)particular attention was required to set up the AS800 injector to ensure the extent of travel of the syringe needle aligned to the entrance of the 0.32mm retention gap tubing. By allowing the trapped needle to penetrate 0.5mm into the 0.32 column, solvent was directly injected onto the retention gap even though the needle geometry did not allow complete insertion into the column as is normal for on-column needles. Centring of the 0.53mm retention gap pre-tube with the 0.32mm retention gap was achieved using a single piece vespel/graphite

ferrule drilled to accept the two column external diameters. Alignment of the needle to the centre of the 0.32mm column was assured due to the lateral freedom of the needle within the 0.53mm tube, coupled with the degree of taper on the needle. The SGE syringe was specifically selected for this characteristic and replaced the standard AS800 Hamilton syringe.

'Component Quantitation'

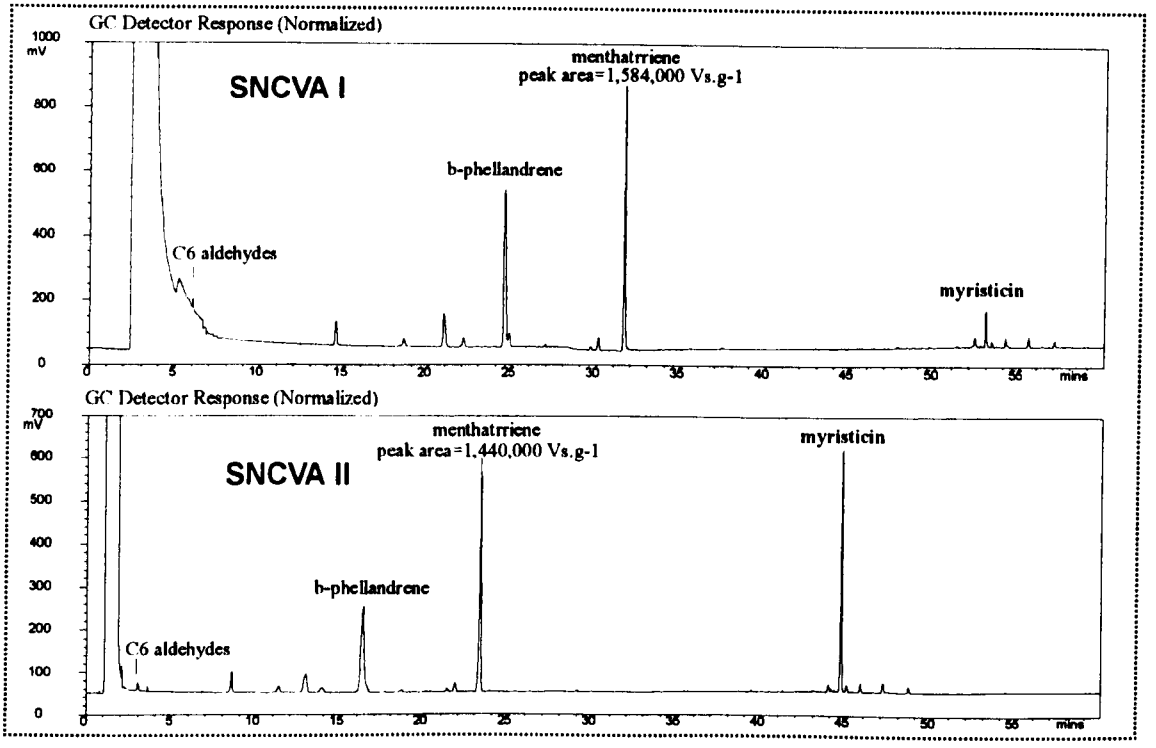
The quantitation protocol described for the SNCVA I technique (Section 3.3.2.1) was applied to SNCVA II. In addition, the flame ionizing detector was calibrated for menthatriene (Section 3.3.2.8) and the following equation used to determine tissue concentration.

$$\text{Concentration (}\ \mu g.g^{-1}\text{frozen parsley)} = \frac{\text{Peak Area (V.s.g}^{-1}\text{ frozen parsley)}}{\text{Quantitation Factor (V.s.ng}^{-1}\text{)} \times 10^3}$$

3.3.2.6 Comparison between SNCVA I vs SNCVA II.

The aims for SNCVA II were to decrease solvent tailing (to permit quantitation of C6 lipoxygenase products), and to improve measurement of high retention compounds, as described in Section 3.1.2. These aims were achieved as can be seen from the chromatograms of the respective techniques (Figure 3.13). Considerable improvement to the dynamics of the solvent peak, in SNCVA II, allowed the C6 products of lipoxygenase to be readily resolved. However, the increase in gas velocity, reflected by shorter retention times of the monoterpenes appeared to compromise the resolution of the system slightly. For example, SNCVA I partially resolved β-phellandrene from the small peak eluting at higher retention time, whereas SNCVA II displayed coelution. From the chromatograms presented, the high retention compound, myristicin, showed significant improvement. However, it was shown later that the improvement in myristicin was not due to the developments within SNCVA II. The issue of myristicin variability is believed to be due to the status of the retention gap in terms of it's slow contamination and subsequent increased affinity for low boiling compounds. Certainly, in this comparison, the SNCVA I retention gap had been repeatedly injected/washed over a six month period whereas the SNCVA II retention gap was new. Finally, the most significant development achieved with SNCVA II was the total automation, allowing multiple samples to be analysed unattended, and eliminating human error from a difficult and labour intensive protocol.

Figure 3.13 Comparison between SNCVA I and SNCVA II for Frozen Parsley.



3.3.2.7 Evaluation of the Reproducibility of the SNCVA II Technique.

To establish the reproducibility of the technique, 24 replicate analyses were performed from a common sample, (1994 harvest stored at -65°C). The chromatographic peak area for the eight largest peaks were determined (Section 3.3.2.1) and analysed using univariate statistics (SAS Institute Inc.) Peak retention times, over the range 14 - 37 min showed excellent reproducibility, with a mean standard deviation of 0.065 min (Table 3.2). Relative retention indices data (Kovats 1958) have not been determined, however the consistency of peak retention times permits the qualitation of components within this thesis to be made with confidence. Preliminary peak area distribution plots, for each of the peaks establish a normal distribution with little or no skew apparent. With the exception of myristicin, mean peak area values varied over a 44 fold range, from 1,512,746 to 34,655 V.s.g^{-1}frozen parsley with a mean coefficient of variance of 8.04% (Table 3.2). From these data, and considering the number of stages involved in the analysis plus the sampling error associated with assaying a natural product, the coefficient of variance is well within acceptable limits. Myristicin, a highly retained compound, has

been observed to display variability during development experiments and has been the subject of development criteria. Observations to date showed that the myristicin peak declined with continual use of the technique over a period of 4 - 6 months, (Figure 4.1, Figure 3.13), as discussed earlier in this section when comparing SNCVA I with SNCVA II. Within this experiment, myristicin analysis displayed a coefficient of variance of 21.6% about a mean peak area are of 142,612 V.s.g^{-1}frozen parsley (Table 3.2).

Table 3.2 SNCVA II Reproducibility Statistics (1994 Harvest)

SAS Institute Inc System

SNCVA Reproduciblity Data

Retention Time (min) t_R	SD	Variable	N	Mean Peak Area V.s.g^{-1}	Std Dev V.s.g^{-1}	%CV[1]	Conc^{n}[2] μg.g^{-1}
14.54	0.067	a-pinene	24	64296	5965	9.28	19.76
16.56	0.069	b-pinene	24	34655	3477	10.03	10.65
17.28	0.063	myrcene	24	122987	9728	7.90	37.79
17.94	0.067	a-phellandrene	24	58281	4115	7.06	17.91
19.17	0.073	b-phellandrene	24	945054	64936	6.87	290.37
22.29	0.067	terpinolene	24	74915	5341	7.13	23.02
23.32	0.060	menthatriene	24	1512746	121361	8.02	464.80
37.32	0.053	myristicin	24	142612	30744	21.56	43.82

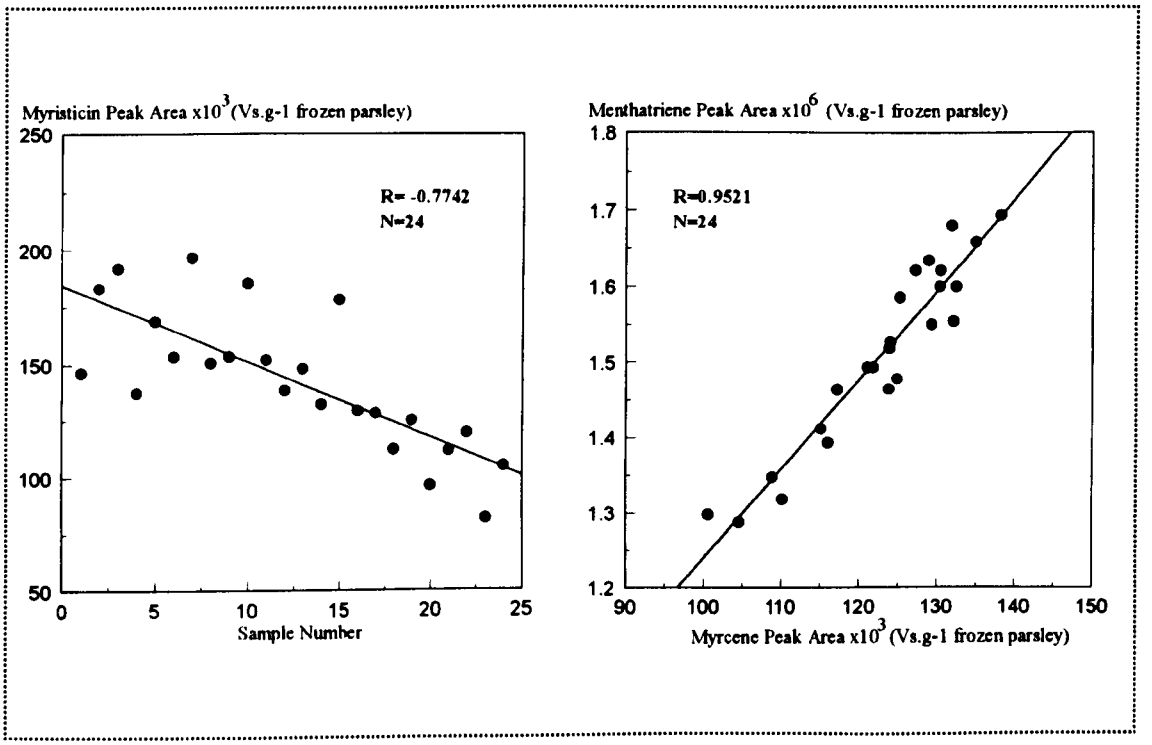
mean peak area %CV (excluding myristicin)= 8.04

1. %CV = coefficient of variance (Std Dev x 100/ mean)
2. Based on calibration for menthatriene (section 3.3.2.8)

Interestingly by plotting the myristicin peak area against sample number, where 'sample 1' is the first sample analysed and 'sample 24' the last, a negative linear correlation coefficient (R = 0.7742, Figure 3.14) can be noted, consistent with the decline noted during repeat usage of the technique. Similar statistical treatment of the other components listed in Table 3.2 does not show significant correlations. The decline of the myristicin peak is believed to be due to slow contamination of the retention gap and its subsequent increased affinity for low boiling compounds. Build up of contamination may be a result of washing the retention gap after the 200°C GLC separation period where polymerisation

of lipids may occur. Such a polymeric material would be increasingly difficult to remove as part of the standard chloroform/methanol wash sequence, as well as serving to trap low boiling apolar compounds. To date, the criteria for washing efficiency have been based on chlorophyll removal, by observing the colour of eluate as it exits the vent line and is collected on white filter paper. The majority of pigmentation deposits after the first rinse with only a small discolouration with the second wash injection and no discolouration on the third injection.

Figure 3.14 SNCVA II Reproducibility Statistics.



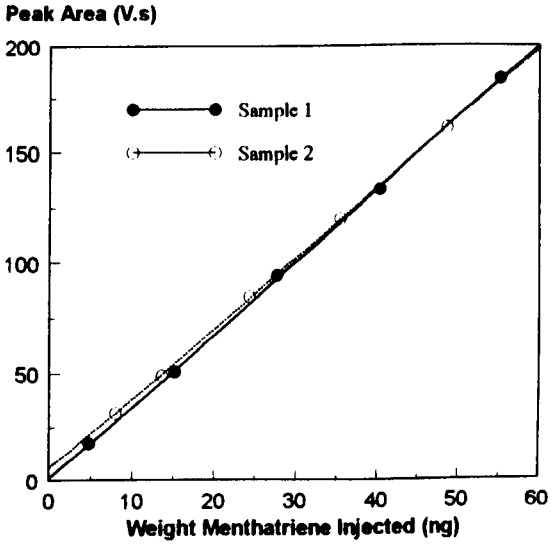
Finally, the variance data calculated for the peaks, other than myristicin, can be shown to contain a systematic error. By plotting the peak area of one peak within an analysis with that of a second peak, for all 24 analysis, a consistent positive correlation can be established, as demonstrated for myrcene against menthatriene (R = 0.9521, Figure 3.14). By creating a correlation matrix (7 x 7 for all 7 peaks, excluding myristicin), 49 linear correlation coefficients can be determined for the data set. The mean of this data set, R = 0.8652, and the coefficient of variance, CV = 5.92% establishes the consistency of this trend for all peaks within a analysis. Self correlation coefficients ie R = 1.000 are excluded from the analysis ie N = 42. From these data it can be shown that if one peak

is high in a particular analysis then all peaks within the same analysis are high. The inference from this information is the existence of a common factor increasing the variance of the data. Such a factor, consistent with this trend might be, for example, the volume of solvent injected and provides a focus for method development with the potential for improving the existing coefficient of variance (CV=8.04%).

3.3.2.8 Detector Calibration for Menthatriene

From a graphical plot of menthatriene peak area against weight of menthatriene injected, a linear relationship was established. Regression analysis, for both injection sequences, was performed and the calibration factor determined from the computed gradient (Figure 3.15). Menthatriene quantitation factor = 3.2545 $V.s.ng^{-1}$.

Figure 3.15 Detector Calibration for Menthatriene.



Regression Output: Sample 1	
Constant (Y axis intercept Vs)	1.1795
Std Err of Y Est	1.2959
R Squared	0.9997
No. of Observations	5
Degrees of Freedom	3
X Coefficient (m = V.s.ng-1)	3.3048
Std Err of Coef.	0.0324

Regression Output: Sample 2	
Constant (Y axis intercept Vs)	5.9302
Std Err of Y Est	0.7740
R Squared	0.9998
No. of Observations	5
Degrees of Freedom	3
X Coefficient (m = V.s.ng-1)	3.2042
Std Err of Coef.	0.0235

Mean Quantitation Factor (Vs.ng-1)	3.2545

3.3.2.9 Identification of GLC components.

The approach used to identify the key compounds within the SNCVA chromatogram involved pre-screening a number of commercial parsley leaf oils to select a composition closest to a typical SNCVA fresh frozen parsley profile. This sample was assayed separately, using GC/MS and SNCVA instrumentation with a common GLC oven profile and chromatographic liquid phase. The qualitative MS data was transcribed onto the parsley leaf oil SNCVA chromatogram using magnitude and retention information. Finally by overlaying the parsley leaf oil chromatogram with a typical SNCVA fresh frozen parsley chromatogram, the original MS data can be further transcribed. Table 3.3 summarizes the mass spectra library search of spectra taken from the main peaks within the TIC chromatogram for parsley leaf oil (Figure 3.16).

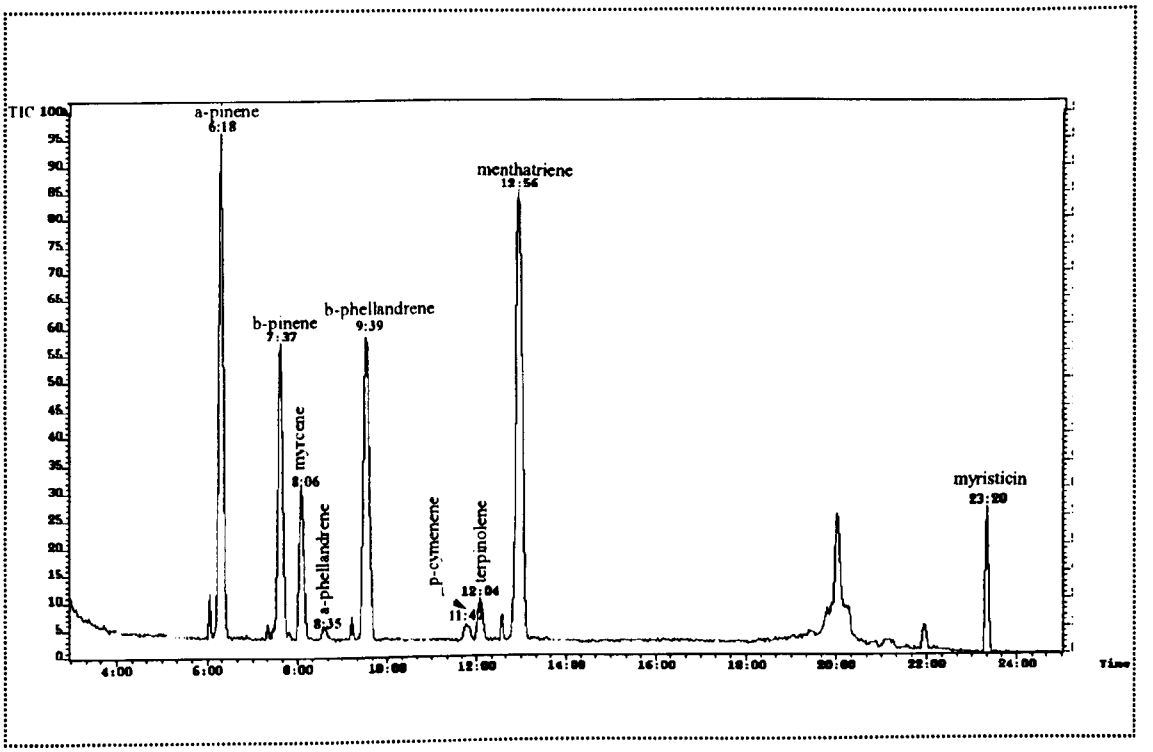
Table 3.3 Summary of Mass Spectra Library Search

Retention Time min:sec	Search Ranking	Library Source	FIT		Chemical Name	CAS
			Purity	Mixture		
6:18	1	NISTREP	987	988	α-pinene	80-56-8
7:37	1	NIST	993	993	β-pinene	127-91-3
8:06	1	NIST	970	970	myrcene	123-35-3
8:35	1	NIST	945	945	α-phellendene	99-83-2
9:39	1	NISTREP	926	930	β-phellendene	555-10-2
11:47	1	UNILEVER	963	964	p-cymenene	1195-32-0
12:04	1	NISTREP	942	943	terpinolene	99-86-5
12:56	1	NIST	932	935	menthatriene	21195-59-5
23:20	2	NIST	990	990	myristicin	607-91-0

The 'purity' value, generated by a forward search across the target peak and the 'mixture' value by a reverse search, allow an estimate of the degree of fit, ideal match = 1000, and whether the target peak is a single compound or a mixture. 'Purity' values significantly below 'Mixture' values indicate a coeluting peak whilst comparable values suggest a single component peak. Examination of the data in Table 3.3 establish both high and

comparable values for these criteria thus increasing the confidence in the identity of the compounds. β-Phellandrene displayed the lowest absolute fit value with the greatest difference between 'purity' and 'mixture' of the peaks tabulated. From earlier data comparing the SNCVA I and SNCVA II methods, (Figure 3.13), a small partially resolved peak following the β-phellandrene peak (RT = 24.7 min) was identified and contrasted with the single peak in the SNCVA II. This peak, tentatively assigned as limonene, would be consistent with the fit values recorded for β-phellandrene, as generated using the SNCVA II protocol. Figure 3.16 and 3.17 show the chromatographic profiles of the three analyses described above, in a format that allowed a comparison and alignment of chromatographic peaks. From the data, good peak alignment was obtained allowing the qualitative MS information to be transcribed onto the chromatogram generated by the SNCVA method for frozen parsley.

Figure 3.16 GC/MS Total Ion Chromatogram of Parsley Leaf Oil



The principal compound in fresh frozen parsley is menthatriene. Figure 3.18 records the mass spectra of this compound generated from the analysis of parsley leaf oil and compared with the matched library standard along with similar data for the related compound p-cymenene. The relevance of p-cymenene inclusion is discussed in Chapter4.

Figure 3.17 SNCVA of Parsley Leaf Oil and Frozen Parsley

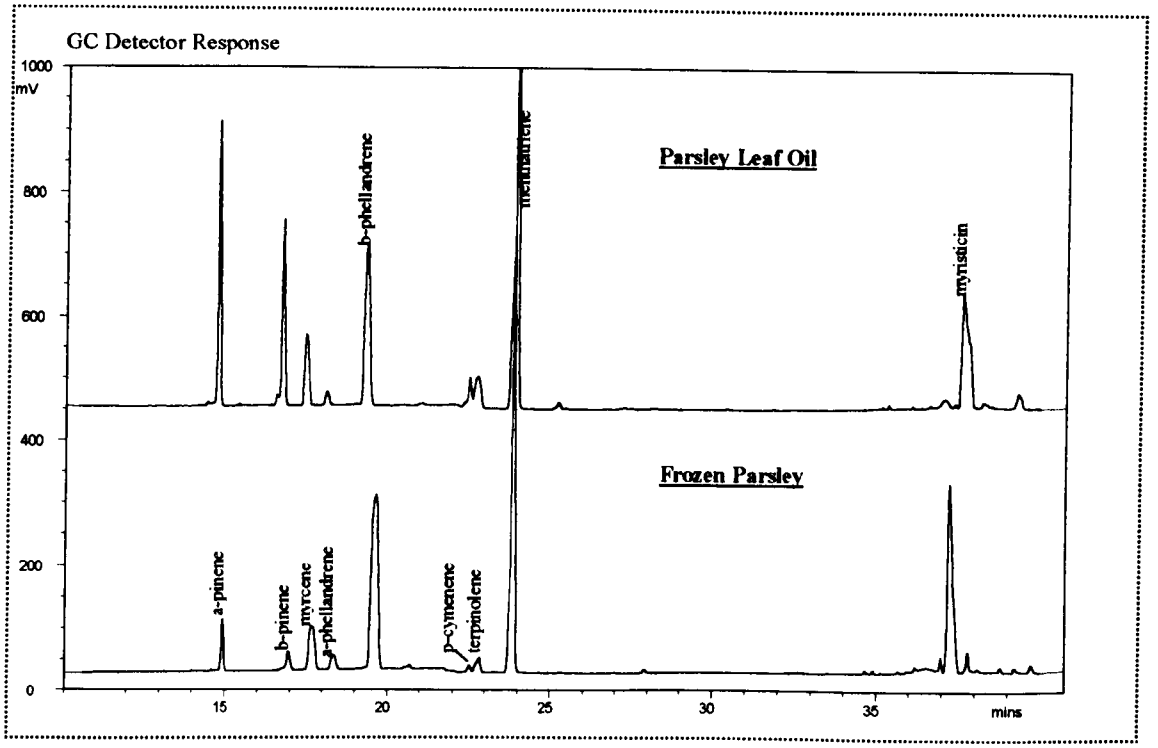
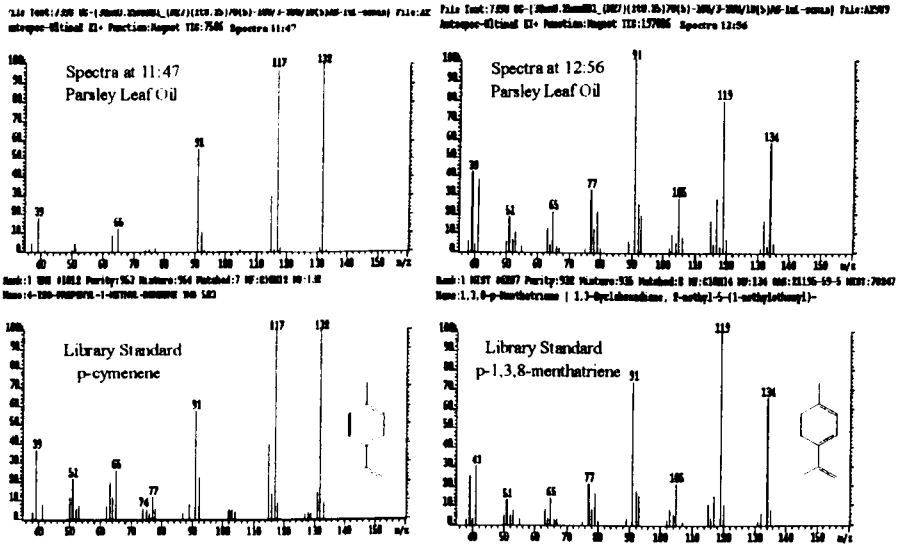


Figure 3.18 Mass Spectra of Menthatriene and p-Cymenene Identified in Parsley Leaf Oil



'SNCVA summary'

SNCVA I was the first analytical component developed in this study as part of the unified strategy for analysis. The method was shown to extract and separate volatile compounds quantitatively from reactive plant systems and provided an analytical capability to generate novel analytical data. The method was applied to parsley, and later developed, (SNCVA II), and combined with non-volatile analysis (SNCNVA).

3.3.3 SNCNVA development and characterisation.

3.3.3.1 Assembly and Set Up of SNCNVA system.

The principal concern in assembling the SNCNVA system was to maintain good chromatographic dynamics throughout the analysis tract. In this respect only pre-cut, end polished tubing was used to avoid dead volumes and the length of transfer tubing and the number of connections were also minimised.

'Component Quantitation'

The Shimadzu photo-diode array (PDA) is integrated with the systems data acquisition computer (LC10) and records absolute absorbance data height (mAbs; area μAbs.s). Absorption spectra for each chromatographic peak was obtained (post run analysis) and used to select a single wavelength at which to quantify the component (Table 3.4). Peak baselines assigned by the systems integration procedure were checked and edited were appropriate, and the area for each peak normalized for the weight of frozen parsley analysed and expressed as the peak area per gram frozen parsley, using the following equation.

$$\text{Peak Area (Abs.s.g}^{-1}\text{frozen parsley)} = A_c \times IPA_\lambda \times \frac{(V^{meth} + V^{chl})}{V_{inj}} \times 1/W$$

where A_c= Absorbance Conversion Factor (μAbs to Abs.s = 10^{-6}).

IPA_λ= Integrated Peak Area at defined wavelength (μAbs.s; Table 3.4)

V^{meth}= Volume of Methanol (10 ml)

V^{chl} Volume of Chloroform (20 ml)

V_{inj} = Volume of Extract Injected (ml) (see Section 3.2.3.1).

W = Weight of Frozen Parsley (g)

Table 3.4 Wavelength Table for Component Quantitation (SNCNVA)

Component	λ_{max} (nm)	Quantitation Wavelength (nm)
chlorophyll 'a'	430	430
chlorophyll 'a' Mg-derivatives	~430	430
pheophytin 'a'	410	410
chlorophyll 'b'	465	465
chlorophyll 'b' Mg-derivatives	~465	465
carotenoids	435-445	440
'flavonoid 1' (apigenin-7-glucoside)	340	340
'flavonoid 2'	340	340
'phenolic 1'	302	302
ascorbic acid	245	245

In this equation, no account is made for the volume of liquid, contributed by the parsley sample, to the total extract volume. The potential effect on the concentration of the sample extract (and thus the peak area), based on an 90% water content and comparing two samples at 1.00 g and 2.00 g (target weight=1.50g), would be of the order of 2-3%.

3.3.3.2 Optimisation of Conditions for SNCNVA Analysis.

The analysis of a model phenolic/flavonoid mixture (Figure 3.19), and the previously assayed, C_{18} reversed phase test mix (data not shown), showed excellent chromatographic peak symmetry and confirmed the workability of a complex/switched analysis path, central to the SNCNVA method. Progressive adjustment of the valve switching times was used to selectively 'heart cut' components of interest from a mixture, as shown for apigenin-7-glucoside from a model 3 component system (Figure 3.19). Spectra generated by the photodiode array detector for each compound are given.

The analysis protocol was extended to the full SNCNVA sequence (Section 3.13). The conditions used for the analysis were developed from the repeated analysis of frozen parsley. Examples of typical chromatograms for the three analyte categories defined, namely ascorbic acid, phenolic/flavonoids, and chlorophyll/carotenoids are shown for frozen parsley stored at -65°C (Figure 3.20 - 3.22 respectively).

Figure 3.19 SNCNVA Chromatography Characteristics and Column Switching Timings for Phenolic/Flavanoid Model System

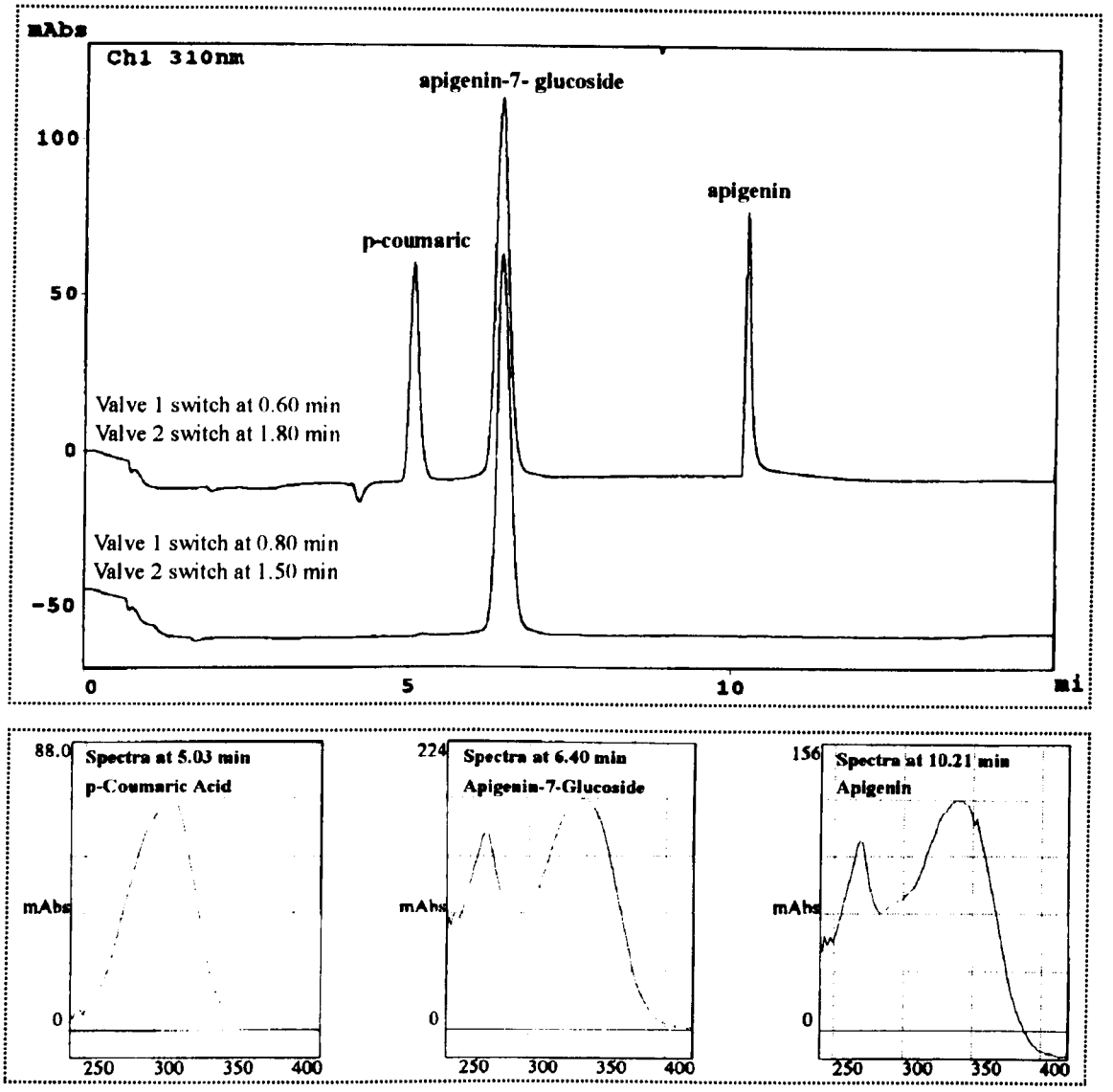
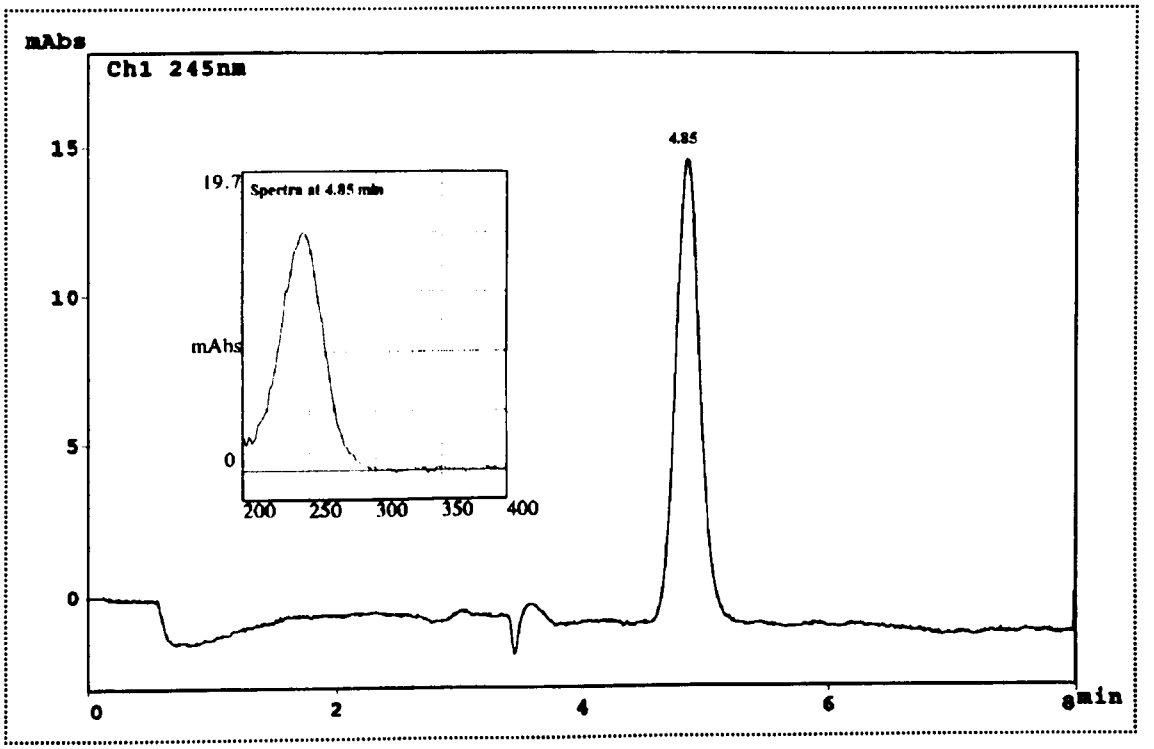


Figure 3.20 shows the chromatogram for the 'acid components' with good peak symmetry for ascorbic acid. The spectrum of the peak (eluted at 4.85 min) shows the characteristic spectra for ascorbic acid with λ_{max} at 245nm. The absence of other components within this chromatogram, as would be expected under the HPLC conditions used, was due to the fine adjustment of valve switch timing about ascorbic acid. Figure 3.21 shows a complex chromatogram for the phenolic/flavonoid components with a mixture of resolved and

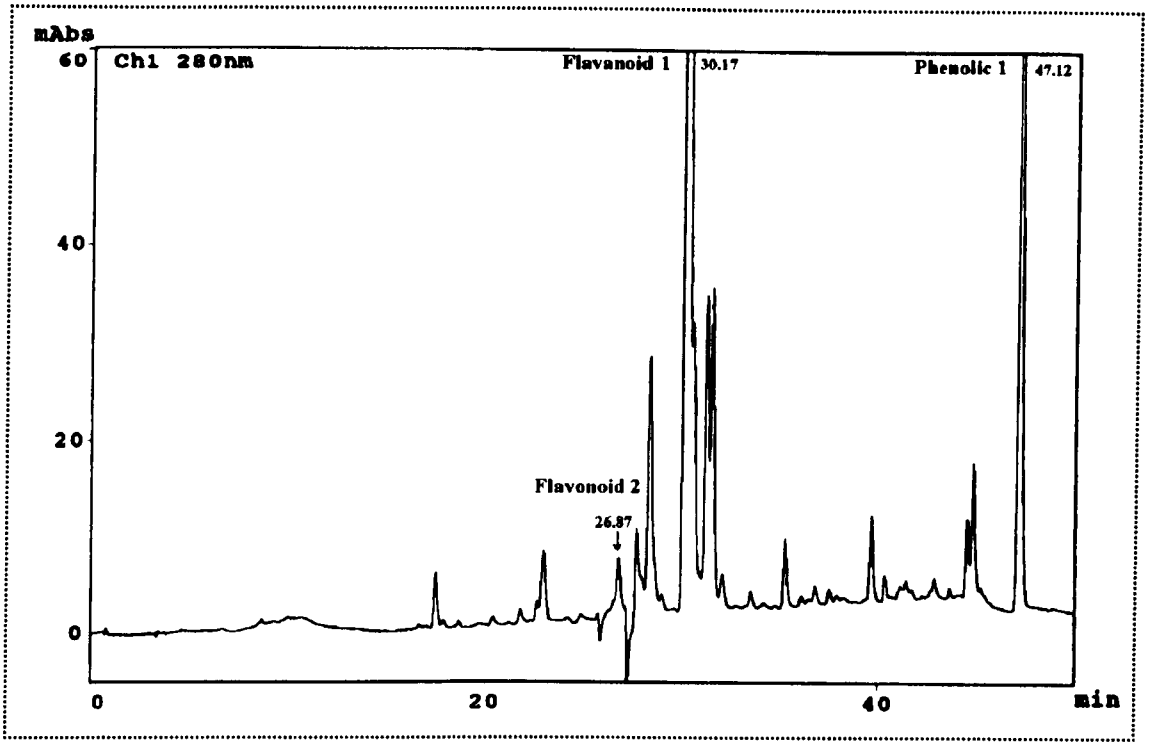
partially resolved aromatic compounds, determined at 280nm. Three 'key peaks' with their associated spectra have been highlighted based on their transient behaviour on frozen storage, as discussed in Chapter 4. Flavonoid '1' was tentatively identified as apigenin-7-glucoside based on its UV spectrum and retention time, as compared to a reference standard for the material. Flavonoid '2', exhibited an almost identical spectra to Flavonoid '1' but eluted at earlier times, suggesting a molecule with increased polarity and not the aglycone, apigenin, expected to elute at longer times with respect to apigenin-7-glucoside.

Figure 3.20 SNCNVA Ascorbic Acid 'Heart Cut' Window



The spectrum shown in Figure 3.21 for 'Flavonoid '2' is taken from a sample of frozen parsley stored at -10°C for 150days rather than 0 days, as is the case for the other spectra associated with Figure 3.21, (note: -65°C storage is defined as equivalent to -10°C for 0 day). This peak increased on frozen storage allowing a better quality spectra to be taken. The final peak identified was an unknown phenolic (Phenolic '1') with retention time 47.12 min and λ_{max}302nm, see Chapter 4. Figure 3.22 shows a typical chromatogram for the separation of carotenoids and chlorophylls. Spectra for these compounds are shown and discussed in the following section of this chapter.

Figure 3.21 SNCNVA Phenolic/Flavonoid Window.



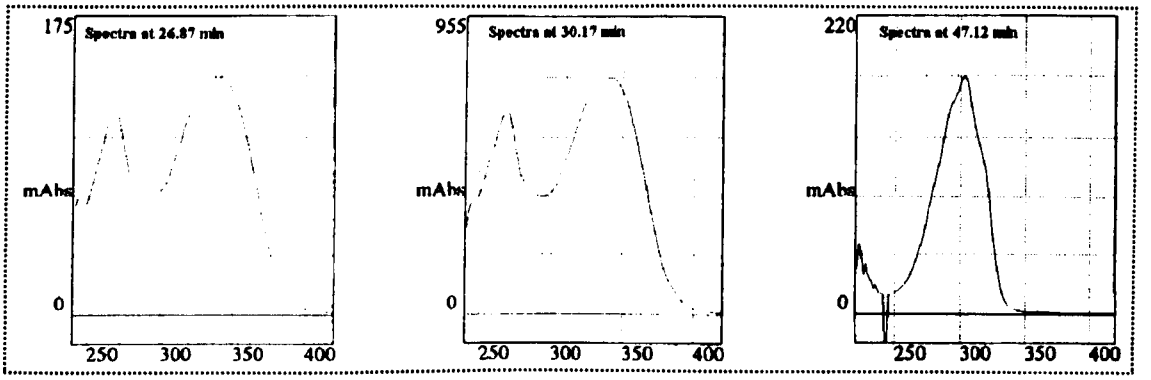
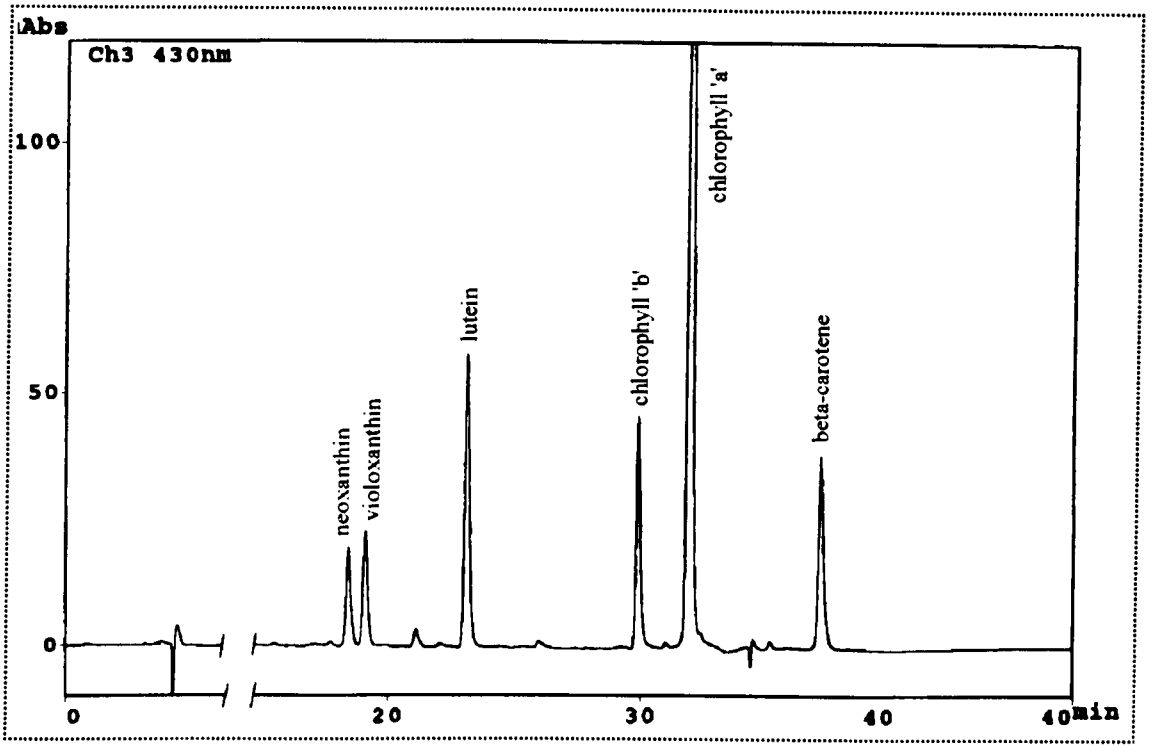


Figure 3.22 SNCNVA Chlorophyll/Carotenoid 'Heart Cut' Window



3.3.3.3 Comparison between SNCNVA and standard acetone extraction.

In a review of literature on the analysis of chlorophylls and carotenoids, the procedure used by Yamauchi *et al.* (1991) was selected, based on its ability to separate the target species along with the numerous chlorophyll degradation products typically encountered in processed leaf tissue. Yamauchi's procedure describes a multiple extraction sequence with aqueous acetone coupled to HPLC separation and photodiode array detection for fresh spinach leaves. This later separation/detection method forms the basis of our chlorophyll/carotenoid analyte window within the SNCNVA system. As part of the SNCNVA development sequence, Yamauchi's method was repeated, utilizing the separation component of the SNCNVA system (single column) for the analysis of fresh spinach and used to validate the data in a comparison with chromatographic data in Yamauchi's paper (Figure 3.23 and 3.24).

Figure 3.23 HPLC Analysis of Chlorophylls and Carotenoids in Spinach

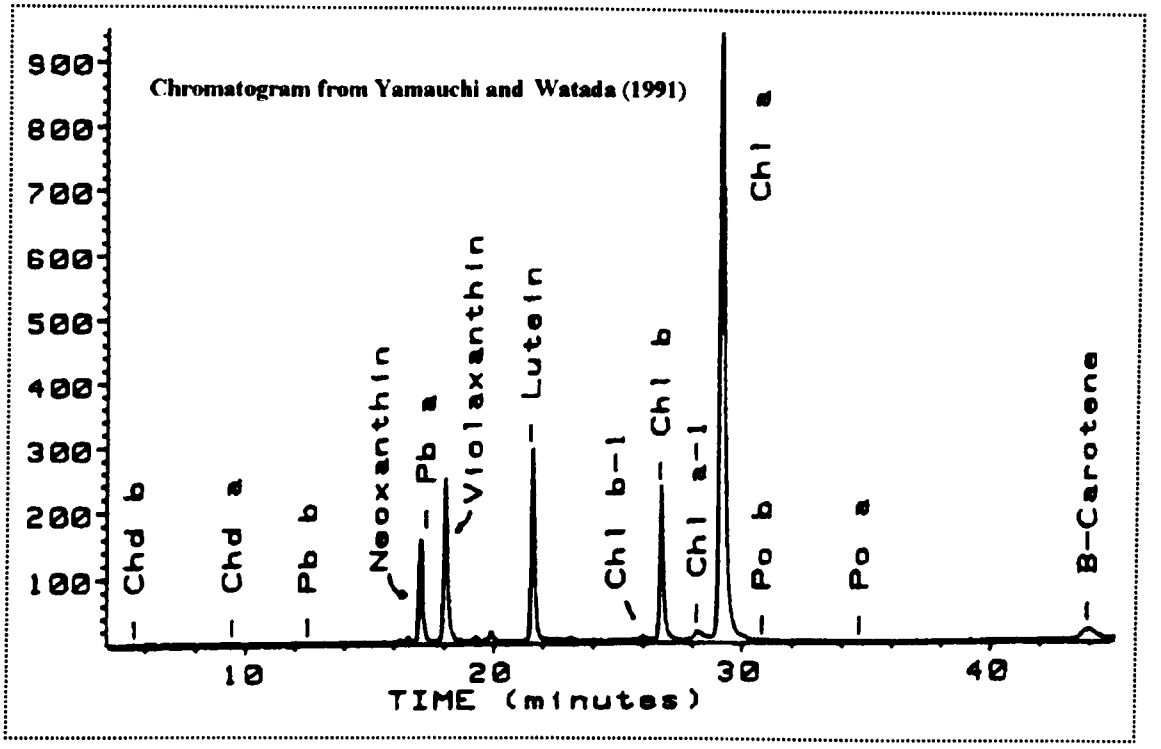
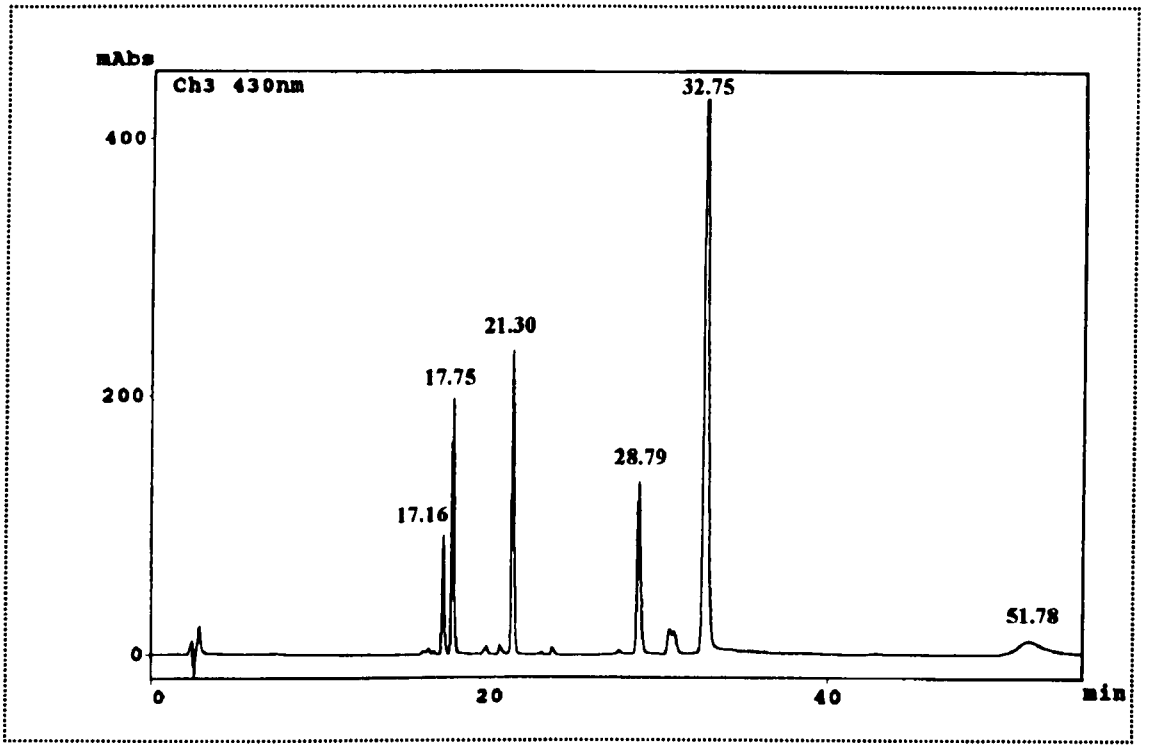
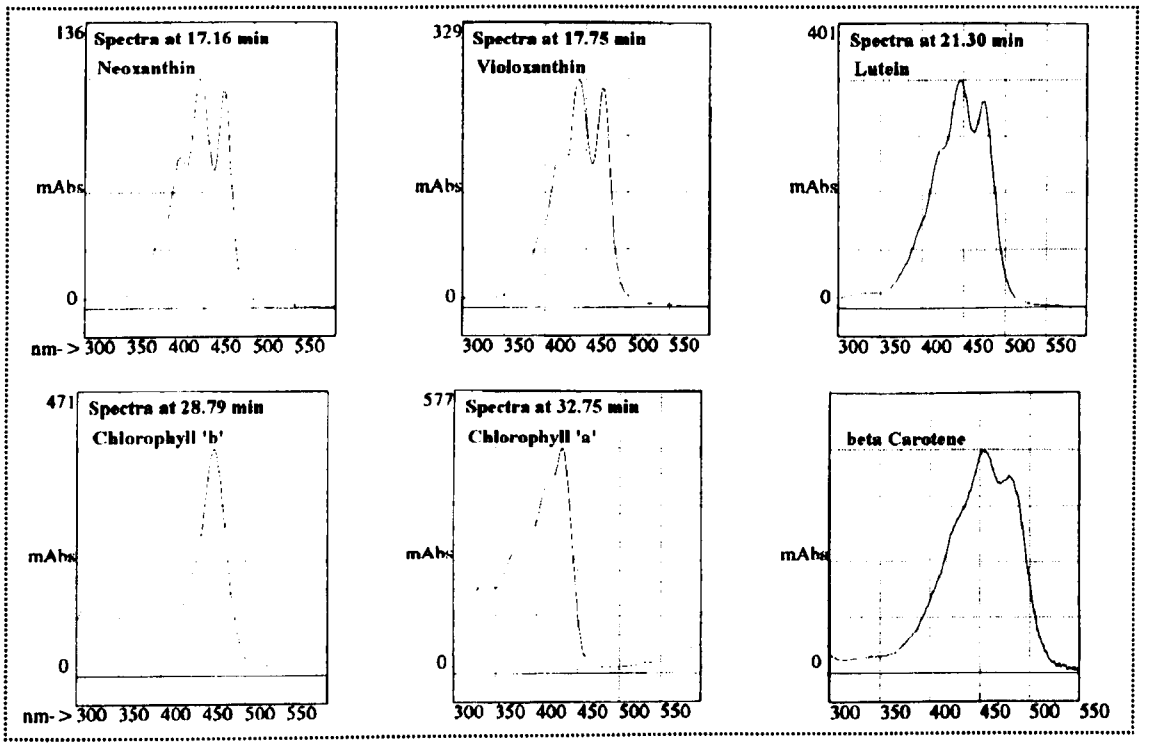


Figure 3.24 'Aq Acetone/SNCNVA of Chlorophylls & Carotenoids in Spinach



From the data, excellent agreement was observed based on comparable peak heights, relative retention times, and common peak spectra (Figure 3.25). The significance of these data is to allow the identification of compounds within the SNCNVA chromatogram and information on the expected retention times and spectra of the primary chlorophyll degradation products namely chlorophyllides, pheophorbides, pheophytins and chlorophyll-

Figure 3.25 Reference Spectra from 'Aqueous Acetone/SNCNVA' of Spinach



13^2-hydroxides (Yamauchi *et al.* 1991).

In the previous experiment, the acetone extraction protocol of Yamauchi *et al.* (1991) was validated against data in the original paper using fresh spinach. The objective to extend this protocol to frozen parsley is to allow a comparison with the newly developed SNCNVA method. In comparing the chromatographic data (Figure 3.26 / 3.27) similar profiles are observed with a number of positive features existing in the SNCNVA data set, which support the design criteria of the method to work with labile analytes. In comparing the chlorophyll 'a' (430nm) to chlorophyll 'b' (465nm) peak area ratio, the SNCNVA method displays a significantly higher value ('chlorophyll peak area ratio$_{SNCNVA}$' = 2.250 versus 'chlorophyll peak area ratio$_{acetone\ extract}$' = 1.605) suggesting the more labile

chlorophyll 'a' species is more highly retained through the SNCNVA analysis procedure.

Figure 3.26 'Aqueous Acetone/SNCNVA' of Chlorophylls and Carotenoids in Frozen Parsley (-10°C 0 Day)

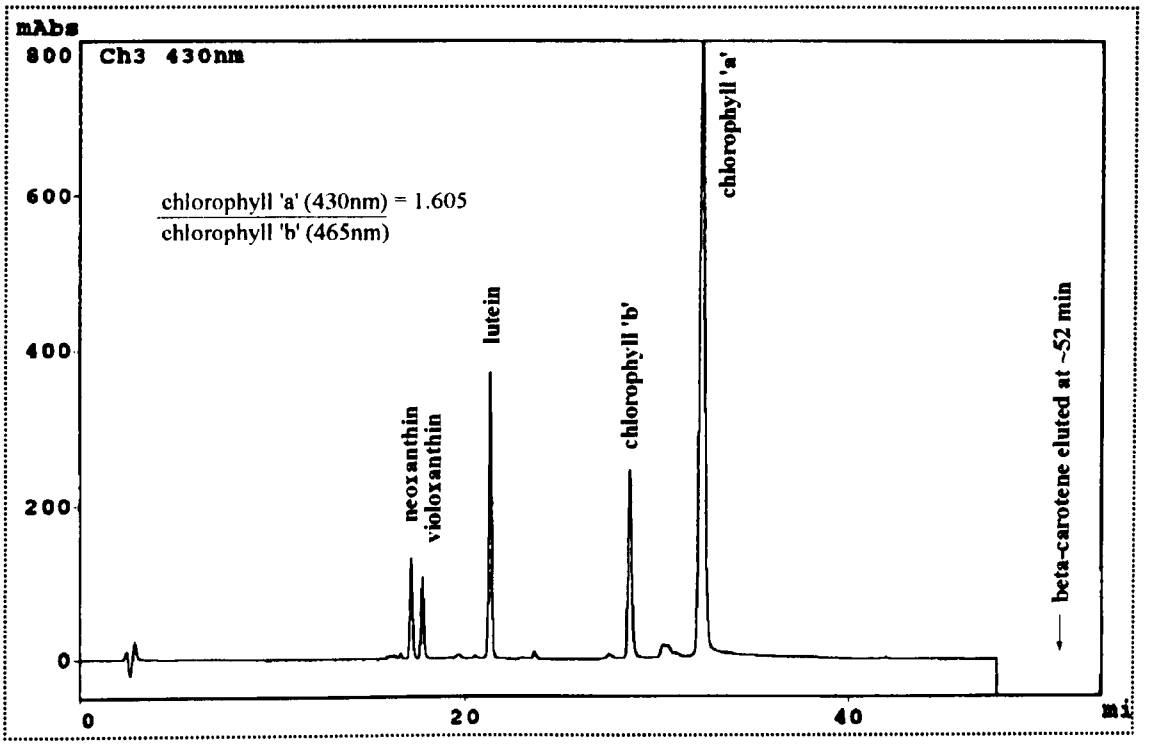
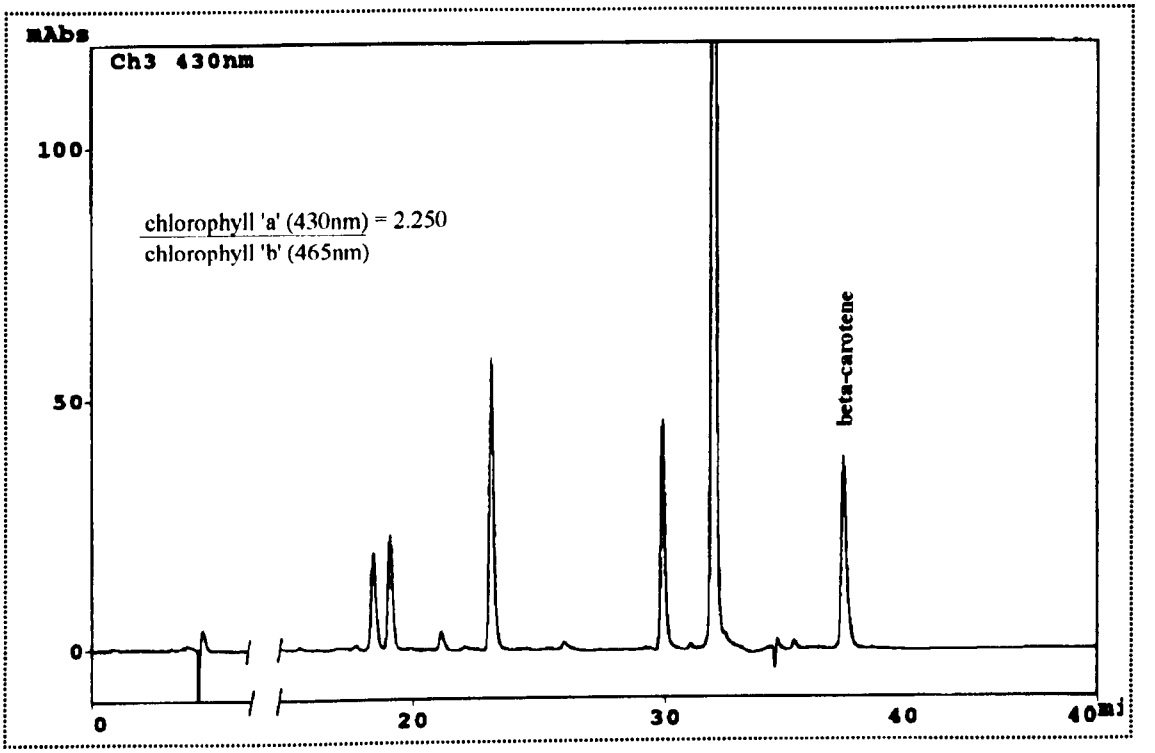


Figure 3.27 SNCNVA of Chlorophylls and Carotenoids in Frozen Parsley (-10°C 0 Day)

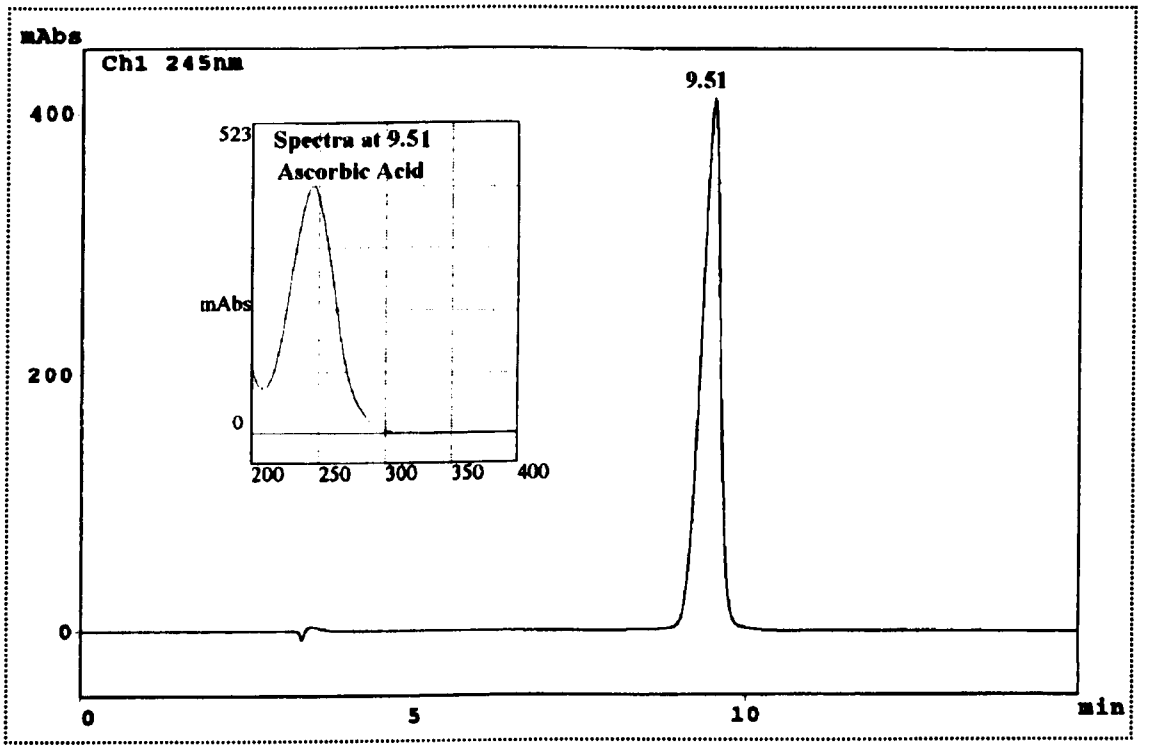


Equally, 13^2-hydroxide chlorophyll (chla -1), a reported degradation product of chlorophyll 'a', eluting between the chlorophyll 'a' and chlorophyll 'b' peaks (RT= 30.34 min) was considerably reduced in the SNCNVA data set. In considering the carotenoids, violaxanthin has been shown to be the most sensitive to degradative change in parsley (see Chapter 4). In this comparison, violaxanthin was found at elevated levels in the SNCNVA data, again tentatively supporting the method's ability to monitor labile compounds. The final improvement shown in these chromatograms was the ability of the SNCNVA method to resolve β-carotene, resulting from the use of a ternary solvent gradient (Figure 3.27).

3.3.3.4 Ion Pair Chromatography for the Analysis of Ascorbic Acid

Development experiments using ion-pair chromatography as a means to increase the capacity factors for low pKa acids proved unsuccessful when the standard SNCNVA chloroform/methanol extraction liquor was injected directly.

Figure 3.28 SNCNVA of an Ascorbic Acid Model System using Ion Pair Chromatography



Typical peaks displayed multiple maxima and considerable tailing, thought to be due to the chloroform disrupting the ion-pair phase. As a development sequence, a model ascorbic acid solution in chloroform/methanol/water was 'phase separated' by the addition of an acidic aqueous buffer to produce two equal layers. The upper layer comprised methanol/water with the polar solutes and the lower chloroform layer contained apolar solutes. Analysis of the upper layer using ion pair chromatography allowed the successful analysis of ascorbic acid with increased capacity factors over standard C_{18} reversed phase chromatography (Figure 3.20 and 3.28). Although this complementary 'phase separation' approach has not been included in this program, potential exists to exploit this area in the analysis of low pKa acids.

'SNCNVA summary'

The novel SNCNVA method appeared to offer significant advantages over traditional multiple extraction methods in allowing the analysis of labile analytes. The method also allowed for the analysis of a large range of analyte groups containing both targeted and untargeted analytes from a single extract sample within an automated sequence. These benefits are complemented by the potential to perform intra analyte correlation, across a range of samples, as a means to identify compounds which may be related to a common reaction sequence. This potential plus the technique's ability to monitor labile analytes should allow for the generation of novel data.

3.4 Conclusions

Within this chapter a unified strategy for the quantitative analysis of volatile and non volatile species has been developed and implemented, SNCVA/SNCNVA, permitting an extensive range of analytes to be measured from a single sample of plant leaf tissue. Characterization experiments using sweet marjoram, spinach and parsley established the technique's capacity to monitor labile species with minimal artifact formation when compared to the traditionally employed protocols of steam distillation and solvent extraction. This strategy is novel to this programme.

Chapter 4

Effect of Frozen Storage Protocols on Flavour Quality Chemicals in Parsley

4.0 Introduction

In Chapter 1, frozen unblanched parsley was selected for investigation because of the extent of quality deterioration during commercial frozen storage. Aroma quality was shown to decline more rapidly than colour quality however, little information on the compounds related to aroma change was available. Colour quality loss was linked to chlorophyll degradation with the possible involvement of peroxidase operating through an oxidative couple with endogenous phenolic compounds capable of the further oxidation of endogenous compounds. A comprehensive review of the volatile and non-volatile compounds in parsley identified important compounds related to both quality and oxidative change. In Chapter 3, novel methodology was established to permit the quantitation of these chemicals under a unified analysis protocol designed to minimise artifactual change and to represent in-tissue concentrations. The unified approach permits the analysis of a wide range of volatile and non-volatile compounds from a single sample, and provides a unique profile of the chemical status of leaf tissue, targeted towards the key chemical and biochemical processes identified in parsley. The method provides a unique opportunity to follow the effect of a specific processing variable (for example frozen storage) by determining the chemical dynamics for a range of compounds and to identify potential relationships between such compounds through statistical correlation. In this chapter, the developed SNCVA/SNCNVA methods are applied to unblanched parsley to establish the effect of frozen storage stress on the chemical dynamics of key endogenous chemicals. Interpretation of the data aims to identify the basis of change, through chemical or biochemical processes (enzymic), by initially considering the chemical functionalities of the compounds degraded and generated. The chemical kinetics for an individual compound and, statistical correlations between compounds are determined as a means to identify potential reaction pathways. Finally qualitative relationships between chemical compounds and quality change are considered.

4.1 Experimental

4.1.1 The Effect of Frozen Storage (-10°C) on the Total Volatile Composition of Frozen Unblanched Parsley (1992 Harvest).

SNCVA I analysis was performed on ~1.5g unblanched frozen parsley (1992 harvest) stored at -10°C for 0 day, 10 days, 30 days, 75 days and 150 days. Figures 4.1, 4.2, 4.4, compare the chromatograms for four storage times and plot the relative areas for the main GLC peaks against storage time (Table 4.1).

4.1.2 Effect of Thawing on the Volatile Composition of Frozen Parsley.

SNCVA II analysis was performed on 3 separate samples of frozen parsley (1993 harvest) stored at -10°C for 0 days (≡ -65°C storage). Samples 2 and 3 were analysed in a slightly modified sequence allowing each sample to thaw from -65°C to 20°C by immersion in a waterbath, for a 5 minute period within the sealed antivortex vials prior to refreezing to -65°C. The samples were subsequently analysed using the standard protocol with the inclusion of hexanal and trans-2-hexenal in the aliquot of methanol added to sample 3. Figure 4.5 compares the three chromatograms in the region representing elution of C_6 compounds.

4.1.3 The Effect of Frozen Storage (-10°C,-20°C) on the Total Volatile, Ascorbic Acid, Flavonoid/Phenolic and Chlorophyll/Carotenoid composition of Frozen Unblanched Parsley (1994 Harvest).

SNCVA II/SNCNVA was performed on ~1.5g unblanched frozen parsley (1994 Harvest) stored at -10°C and -20°C for 0 day, 10 days, 30 days, 75 days and 150 days. Figures 4.6-4.11 plot the chromatographic peak areas against storage time for the analyte groups.

4.2 Results and Discussion

4.2.1 Effect of Frozen Storage on the Volatiles in Parsley.

The effect of frozen storage (-10°C, 1992 harvest) on the total volatile composition of parsley is compared in Figures 4.1, 4.2. Under unstored conditions, the monoterpene hydrocarbons (myrcene, 63 $\mu g.g^{-1}$; β-phellandrene, 227 $\mu g.g^{-1}$; menthatriene, 275 $\mu g.g^{-1}$) comprise the major volatiles present along with the phenyl propanoid compound, myristicin (172 $\mu g.g^{-1}$; Table 4.1).

Figure 4.1 Effect of Frozen Storage (-10°C) on the Volatile Composition of Parsley.

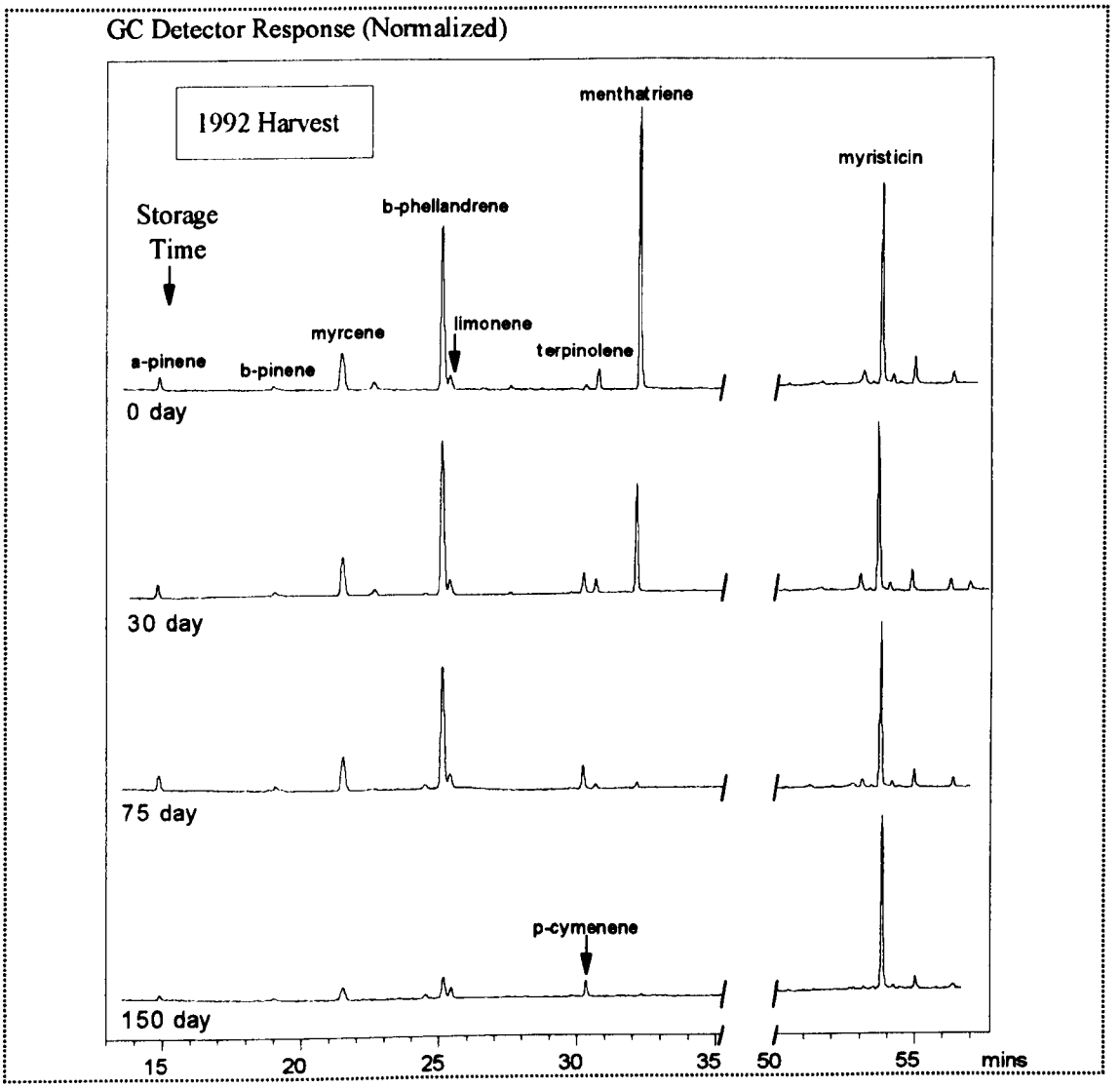


Figure 4.2 Effect of Frozen Storage (-10°C) on the Volatile Composition of Parsley.

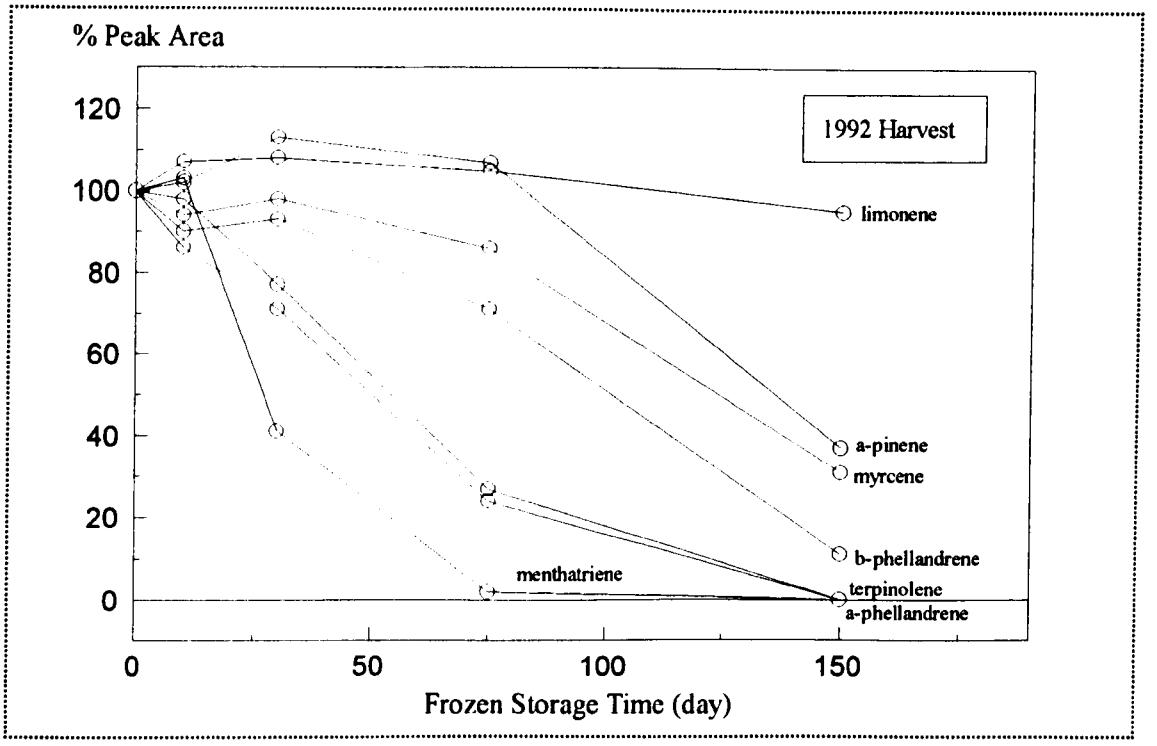


Table 4.1 Effect of Frozen Storage (-10°C) on the Volatile Composition of Parsley

Retention Time (min)	Chemical Name	Concentration ($\mu g.g^{-1}$ frozen parsley) Storage Time (Day)					% Concentration at 0 Day
		0	10	30	75	150	
14.2	α-pinene	14.76	15.05	16.75	15.85	5.49	1.83
18.3	β-pinene	5.34	5.86	6.51	6.54	3.07	0.66
20.8	myrcene	62.66	59.07	61.52	53.91	19.67	7.79
21.9	α-phellandrene	12.69	12.43	9.79	3.40	0.00	1.58
24.4	β-phellandrene	226.61	203.63	210.37	161.81	25.25	28.15
24.7	limonene	11.40	12.23	12.31	12.00	10.87	1.42
29.5	p-cymenene	4.94	7.83	21.62	23.92	14.10	0.61
30.0	terpinolene	20.09	17.27	14.21	4.79	0.00	2.50
31.5	menthatriene	274.47	282.47	113.31	5.26	0.00	34.10
35.6	(menthatriene diepoxide)	0.20	0.37	0.62	1.25	6.19	0.02
52.6	myristicin	171.74	122.34	153.35	138.84	141.86	21.34
	Total	804.90	738.55	620.38	427.57	226.51	100.00

Quantitation was established using a quantitation factor determined for menthatriene (Chapter 3.3.2.8) and applied to the other compounds (assumes comparable response factors). From literature, the levels of essential oil and the chemical composition in parsley leaves vary marked (0.04-0.15%; menthatriene 20-60% respectively; Simon *et al.* 1988) across varieties. From SNCVA data, total volatiles equate to ~ 0.08 % w/w frozen parsley (Table 4.1) and menthatriene comprises ~ 34 %. These values appear consistent with literature.

On frozen storage the monoterpene hydrocarbons appear to degrade at differential rates. Menthatriene displays the greatest rate of change showing almost complete degradation over a 75 day period, whilst limonene remains largely unchanged, (Figure 4.2). To investigate the kinetics of the degradation of menthatriene (1994 harvest), graphical plots of the rate equations (integrated form) for zero, first and second order reactions were generated, (Atkins1983). From these data, a linear relationship for a first order plot, (ln(a/(a-x)) vs time), was obtained indicating the degradation reaction is first order with respect to menthatriene (Figure 4.3, Table 4.2).

Figure 4.3 First Order Rate Plot for Menthatriene.

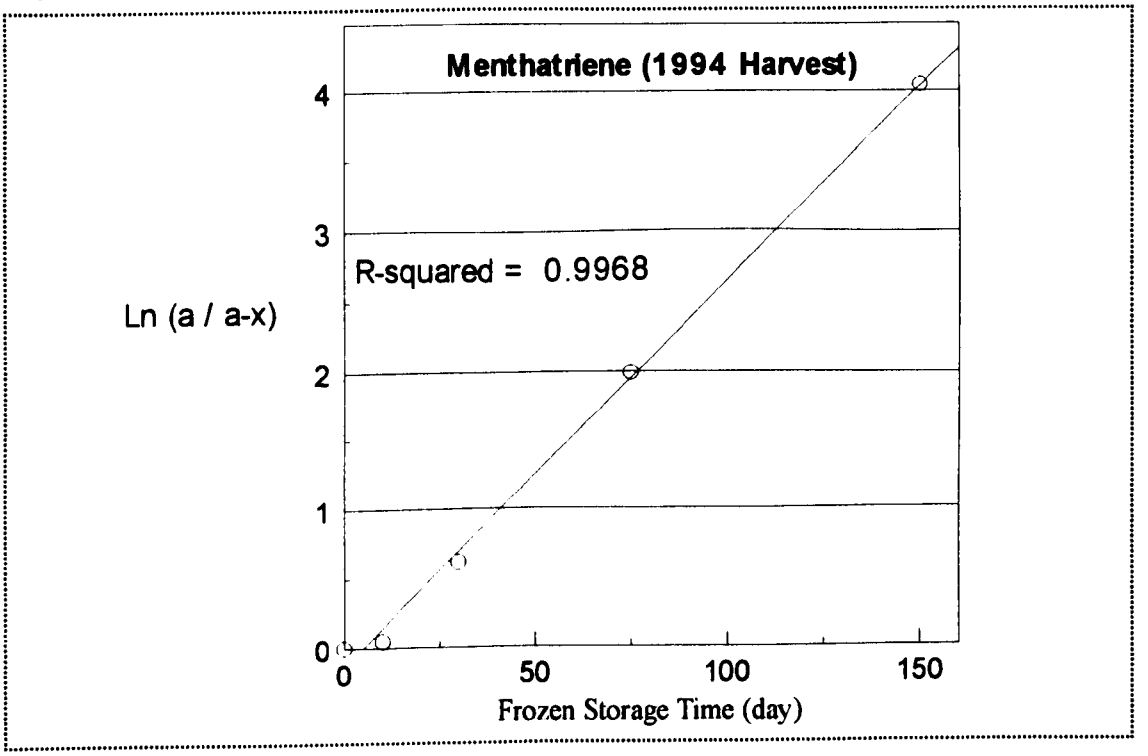


Table 4.2 Reaction Kinectics for Analytes Determined by SNCVA/SNCNVA in Frozen Parsley (-10°C).

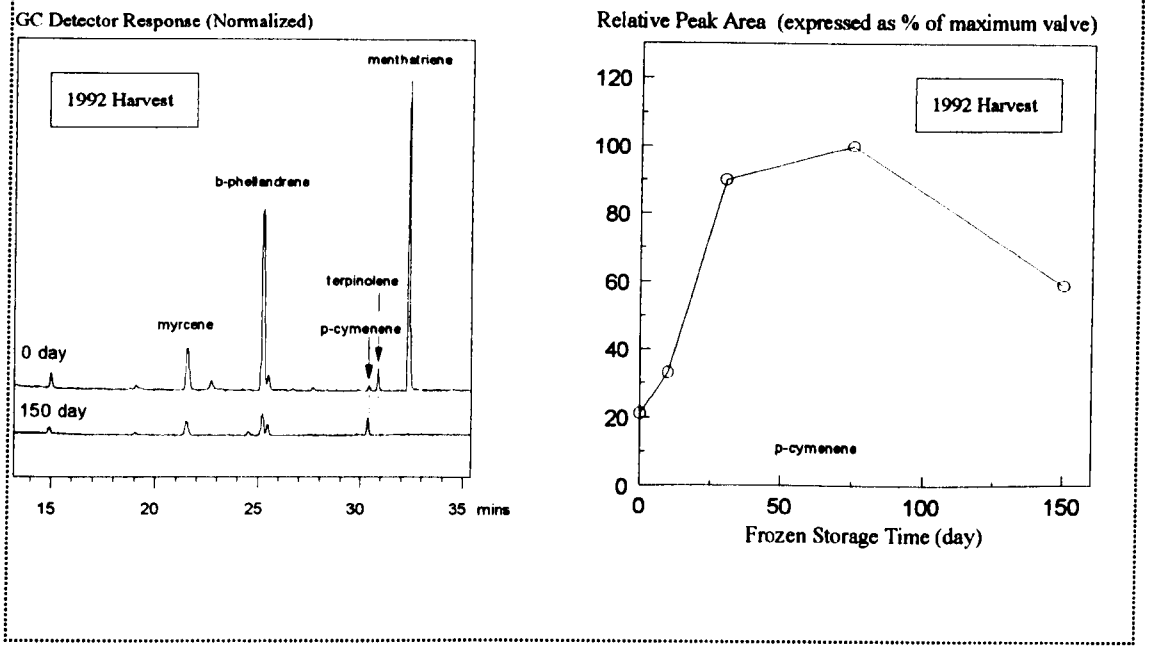
Reaction Kinectics (-10°C) A ---> P	Degradation Reaction (reactant [A])		Formation Reaction (product [P])	
	compound	R^2	compound	R^2
zero order (a-x) vs t	violoxanthin	-0.999	chl 'b' 13^2OH	0.995
	lutein	-0.998	pheophytin 'a'	0.962
	chlorophyll 'a'	0.996	flavanoid 2	0.999
first order ln (a/(a-x) vs t	menthatriene	0.996		
	phenolic 1	0.996		
second order x/a(a-x) vs t	ascorbic acid (-20°C)	0.979		

where a=[reactant] at t=0, a-x=[reactant] at time,t.

Experimental reaction kinetics are useful when formulating a mechanism for a reaction (comprising of a sequence of unimolecular or bimolecular steps) as an acceptable mechanism must conform to the observed rate laws. This information would assist investigation of the basis of change in parsley however it has not been taken further within this thesis.

p-Cymenene, an oxidised (aromatized) form of menthatriene, represents the only significant volatile compound generated over the storage period, however there is no stoichiometric relationship between the loss of the major monoterpenes and the appearance of p-cymenene (Figure 4.4). p-Cymenene also displays transient behaviour over the assessment period (150 day) and would appear to be a reactive compound, potentially formed from menthatriene either as an intermediate or as a secondary side reaction. The importance inference from these data is that the monoterpene hydrocarbons are extensively lost on frozen storage and that the reaction products are sufficiently non-volatile to be excluded from the 'volatility cut' of the SNCVA technique. This apparent decrease in volatility is likely to arise from an increase in molecular weight which may occur due to dimerization/polymerization or the multiple addition of polar functional group.

Figure 4.4 Effect of Frozen Storage (-10°C) on p-Cymenene Level in Parsley.



The second class of volatile compounds found by SNCVA analysis are the phenyl propanoids, represented by myristicin. In contrast to the monoterpenes, myristicin is stable over the storage period (Figure 4.1). From this information the chemical functionalities of the two classes of molecules (monoterpene vs phenyl propanoid) can be compared and contrasted to identify a potential basis for reactivity (Appendix 1.3). It is clear and significant, however, that neither chemical classes, as represented, are known substrates for the oxidoreductase enzymes, particularly lipoxygenase, polyphenol oxidase or peroxidase, and would suggest that the change either involves an alternative enzyme system/mechanism, or is chemical in nature.

In addition to rationalizing the data in terms of chemical/biochemical reactions, two important physico-chemical processes, that of evaporative loss and the partitioning into the material of the storage vessel, need also to be considered. These aspects were examined in the selection of methodology employed (SNCVA/SNCNVA, Chapter 3) and in the experimental design (Chapter 2). The data presented in Chapters 2 and 3 suggests

that neither evaporative loss nor volatile partitioning can account for the changes on storage observed (Figures 4.1).

In the introduction to this thesis (Chapter 1.6.1), the degradation of menthatriene was discussed. Nitz *et al.* (1989; Technical University Munich) referred to the study of Hartmann (1985 PhD thesis; Technical University Munich; language: German) and suggested that menthatriene underwent oxidative loss on frozen storage with the formation of the associated 1,4 endoperoxide and 1,2-3,4-diepoxide. The thesis of Hartmann has recently been translated (commissioned within this study) and forms a significant study which investigates areas of work similar to those conducted within this thesis (volatiles only). Hartmann (1985), identified 1,2-3,4-diepoxy-menth-8-ene in frozen parsley and noted that the formation on storage (-18°C, 8 months) appeared to relate to menthatriene degradation. However it did not follow a stoichiometric conversion (1:1), (menthatriene loss ~40 mg/kg; 1,2-3,4-diepoxy-menth-8-ene increase ~13 mg/kg). 1-Methyl-4-(1-methylethenyl)-2,3-dioxabicyclo[2.2.2]oct-5-ene (1,4 endoperoxide of menthatriene) did not appear to be recorded in frozen stored parsley (Hartmann 1985), contrary to the citation of Nitz *et al.* (1989). p-Cymenene was also not identified to increase in stored frozen tissue, however was quantified with terpinolene, (as a coeluting peak), which showed no pronounced change over 8 month frozen storage. These data on menthatriene degradation from Hartmann's thesis appear to contrast significantly with the data presented in this thesis, whereas comparable results are obtained for the formation of lipid oxidation products on tissue thawing (see Section 4.2.2) and the stability of the phenyl propanoids (eg myristicin) on frozen storage. Two primary issues can be identified as discriminating between the data of this study and that of Hartmann. Firstly, the lack of formation of the 1,2-3,4-diepoxide relative in menthatriene loss, in our data and secondly a similar lack of formation of p-cymenene in Hartmann's data. With respect to p-cymenene, Hartmann utilized a slightly more polar GLC liquid phase (SE54 95% dimethyl, 5% phenyl silicone) than in our study (CP Sil5 100% dimethyl silicone) and observed that p-cymenene coeluted with terpinolene. From our data we observed that terpinolene decreased on frozen storage (-10°C) with a comparable increase in p-cymenene (Figure 4.1; Table 4.1) and therefore may explain why no apparent net change was observed when the components were quantified together as a coeluting chromatographic peak. Additionally, from Hartmann's data, the level of 'p-cymenene + terpinolene' (~5 mg/kg foliage) is shown to

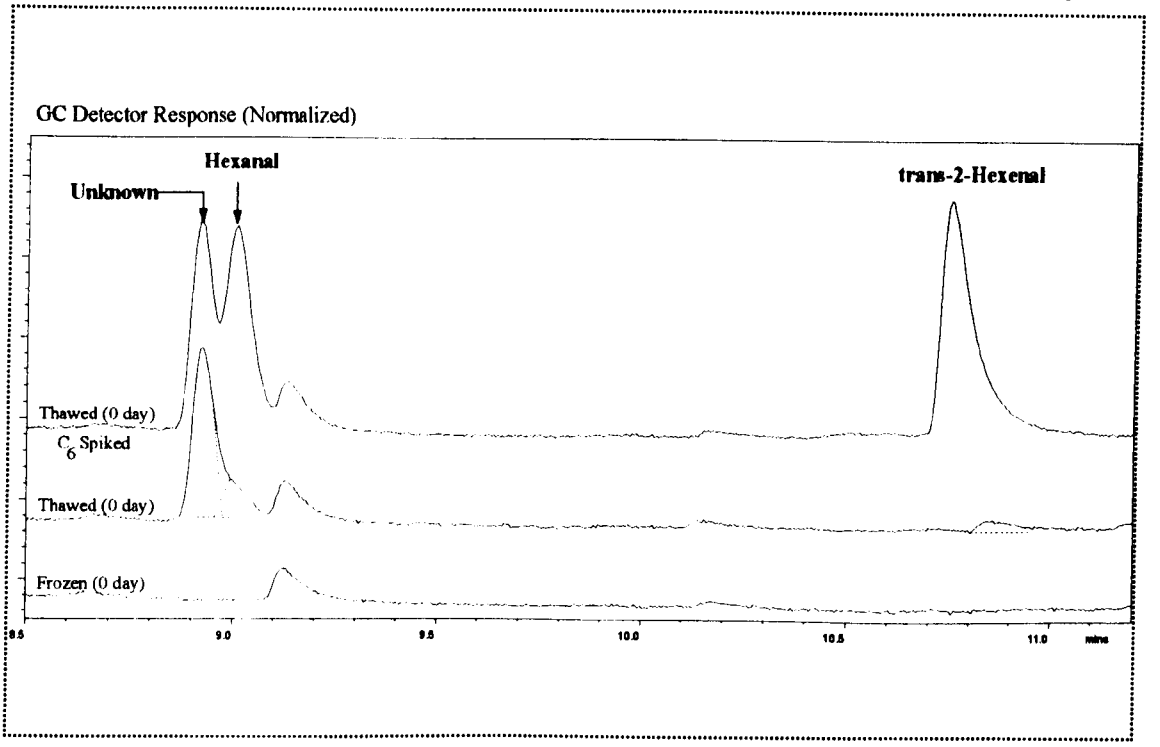
be approximately half that for the 1,2-3,4-diepoxide formed after 8 months storage (13 mg/kg foliage). From these values and the known retention time for the 1,2-3,4-diepoxide (SE54 phase), we would expect to see this degradation product in our chromatographic data. On re-examining our data, we do observe a small unidentified chromatographic peak at a retention time (t_R ; t_R=35.6 min; 1992 Harvest, SNCVA I) similar to that expected from Hartmann's retention data. The peak is in a region of the chromatogram, shown by both data sets, to contain only minor components. From our data, this peak is approximately 2-3 times larger than other peaks in this region of the chromatogram, however it only comprises approximately 5% of the area of p-cymenene (75 day -10°C, Table 4.1) and therefore only represents a minor degradation product. A third discriminating area between Hartmann's data and that of this thesis is the levels of menthatriene (60 mg/kg foliage and 274 $\mu g\ g^{-1}$ frozen parsley respectively), however, this variation may result between different varieties (Simon *et al.* 1988). Further discussion on the basis of volatile change in frozen parsley are made in Chapters 5 (5.2.5) and 6 (6.2.2).

4.2.2 Effect of Thawing on the Volatile Composition of Frozen Parsley .

To date, the information presented relates to frozen tissue, which by design, has not had opportunity to thaw, as would typically occur during the consumption of frozen parsley. The effect of thawing parsley, for a 5 minute period, shows no significant effect on the major volatile compounds (Chapter 5) however, three minor peaks are formed at low retention times (Figure 4.5). Two of these are tentatively assigned as hexanal and trans-2-hexenal and are likely to reflect the action of lipoxygenase, C_{13} hydroperoxide lyase and double bond reducing enzyme/isomerase (Figure 1.9; Eriksson 1979). Interestingly, parsley frozen at -10°C and stored for 10 days did not possess the same capacity to generate these volatile products, (data not shown), and appears consistent with the loss of 'grassy' character on frozen storage, (see Section 4.2.6). In view of the fact that lipoxygenase is a stress enzyme being activated by cell damage, these data suggest that either lipoxygenase is activated by the damage caused by freezing/leaf division at liquid nitrogen temperatures and the volatile products are subsequently degraded, or the direct or indirect substrate for lipxoygenase, in this case linoleic acid/C_{18} lipids are lost by other processes during frozen storage. Certainly the polar lipids in frozen parsley, particularly

the phospholipid are known to degrade in similar timescales (Duden *et al.* 1982, Chapter 1).

Figure 4.5 The Effect of Thawing on the Volatile Composition of Parsley.



The presence of hexanal in parsley tissue has been reported (MacLeod *et al.* 1985, Kim *et al.* 1990) although it was not identified in the work of Jung *et al.* (1992), who studied odour impact chemicals in parsley using aroma extract dilution analysis, see Chapter 1.5.1/1.6.1. This suggests that, although it may be present in parsley, it is not sensorially significant. The third volatile peak, which eluted immediately prior to hexanal, identified as being formed on thawing and again sensitive to 10 day storage is unknown, (Figure 4.5). AEDA analysis showed methyl-2-methyl butanoate eluted in a similar region of the chromatogram (Jung *et al.* 1992), however spiking experiments showed this compound to be eluted between hexanal and trans-2-hexenal. So the identify remains undetermined.

4.2.3 Effect of Frozen Storage on Chlorophyll and Carotenoids in Parsley.

In the previous chapter, the benefits of performing multiple analyte analysis, including both volatile and non-volatile analytes, from a single sample were discussed. Of particular

importance was the potential to perform correlations between an extensive range of endogenous compounds, as a function of frozen storage, and to utilize the relationships established as a means to prompt causation experiments and discussion. Linear correlations (Pearson type 'SAS Institute Inc') between all the quantified compounds determined by SNCVA/SNCNVA analysis have been performed (1994 harvest). Those correlations with high R^2 values were selected (Table 4.3) and are discussed in the following sections.

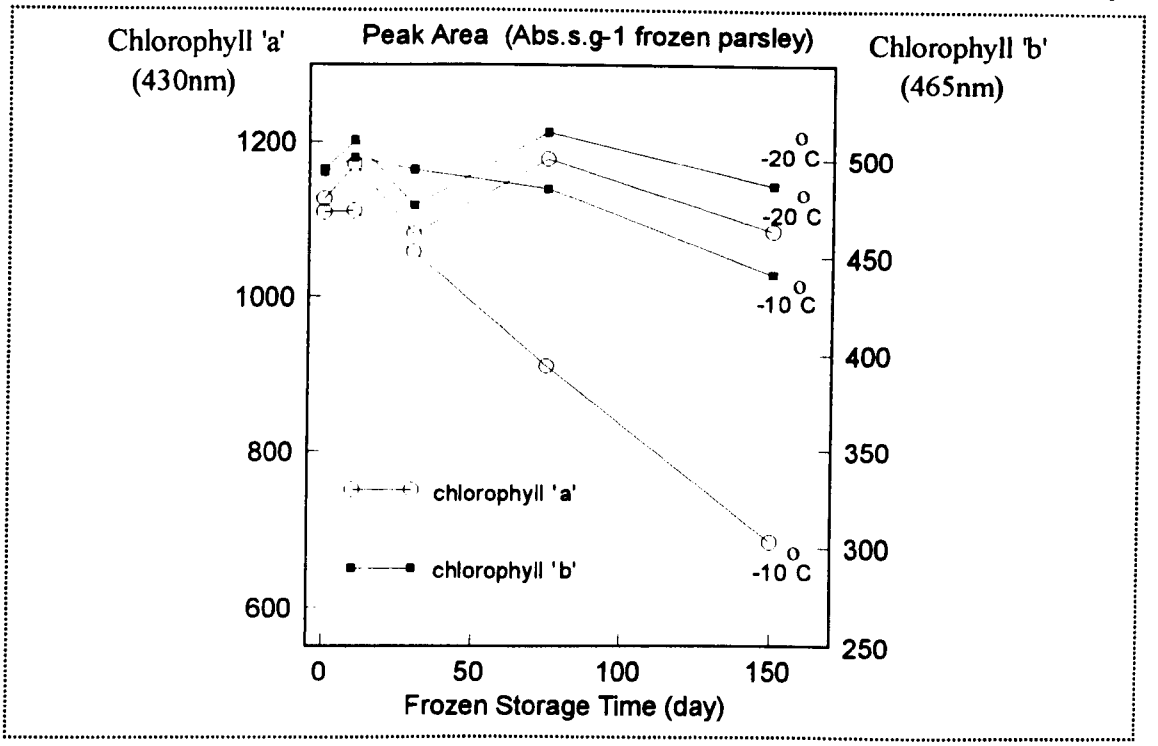
Table 4.3 Linear Correlations between Analytes Determined by SNCVA/SNCNVA Analysis

Variable 1	Variable 2	Correlation Coefficient R^2
Flavanoid 2	violoxanthin	-0.995
	lutin	-0.997
	chlorophyll 'a'	-0.995
	chl 'b' 13^2 OH	0.998
Phenolic 1	chl 'a' 13^2 OH	-0.997
	p-cymenene	-0.963
	menthatriene	0.999
chlorophyll 'a'	pheophytin 'a'	-0.972 gradient1† = -0.24
chl 'a' 13^2 OH	p-cymenene	0.989

† gradient = pheophytin 'a' peak area at 410nm / chlorophyll 'a' peak area at 430nm

The effect of frozen storage on the chlorophyll content of unblanched parsley stored at -10°C showed a significant degradation of chlorophyll 'a' with minor loss of chlorophyll 'b' (Figure 4.6). At -20°C little degradation in either species was observed. These data are consistent with observations from literature that aroma volatiles are more sensitive to degradation on frozen storage than chlorophyllian pigments and that the rate of change is a function of storage temperature (Philippon *et al.* 1986; Figure 1.6).

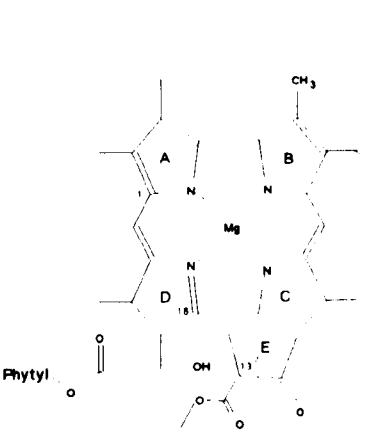
Figure 4.6 Effect of Frozen Storage (-10°C) on Chlorophyll Level in Parsley.



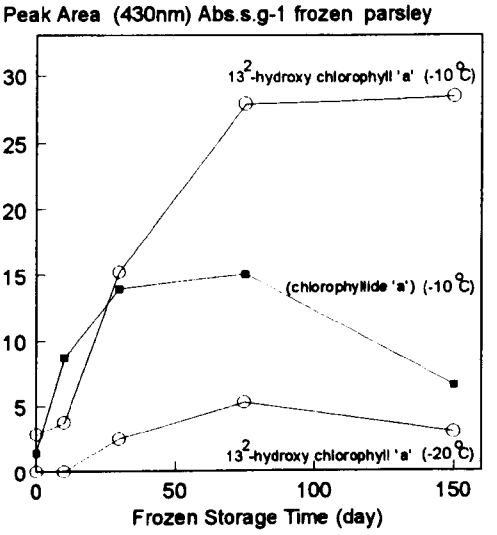
If we consider the kinetics of the loss of chlorophyll 'a' at -10°C we observe an apparent zero order process with respect to chlorophyll 'a', (Table 4.2). Associated to this loss is the correlated (linear) formation of pheophytin 'a' with similar zero order kinetics (Table 4.2 and 4.3; Figure 5.10). From literature, the kinetics of chlorophyll loss have been extensively studied particularly in relation to heat processed vegetable tissue, where the reaction has been shown to involve an acid mediated protonation of the porphyrin ring with loss of magnesium, to form near stoichiometeric amounts of pheophytin under first order kinetics (Schwartz *et al.* 1990). In contrast to this position, unblanched frozen parsley shows pheophytin formation accounts for a significant but minor proportion of the chlorophyll lost, estimated at approximately 24% conversion between the species, based on quantitation of chlorophyll 'a' and pheophytin 'a' at a wavelength of 430nm and 410nm respectively (Figure 5.10). In addition to the formation of pheophytin 'a', two other degradation products of chlorophyll 'a', namely 13^2-hydroxy chlorophyll 'a' and chlorophyllide 'a' are observed to have transient formation characteristics over the storage period (Figure 4.7). 13^2-Hydroxy chlorophyll 'a', the so called allomerization product, is formed by free radical autoxidation, (Hynninen 1981), as well as by the action of

chlorophyll oxidase in a sequence involving activation or coupling with linolenic acid, (Schoch *et al.* 1984). From the chemical functionality of the chlorophyll 'a' molecule (Figure 1.12), it would not be expected to form a natural substrate for the oxidoreductase enzymes, particularly lipoxygenase, polyphenol oxidase or peroxidase (Chapter 1.6.5). In contrast, chlorophyllide 'a' is the product of the enzymic action of chlorophyllase on chlorophyll 'a' which produces the dephytylated form of the molecule.

Figure 4.7 Effect of Frozen Storage (-10°C) on 13^2-Hydroxy Chlorophyll 'a' and Chlorophyllide 'a' Level in Parsley.



13^2-hydroxy chlorophyll 'a'

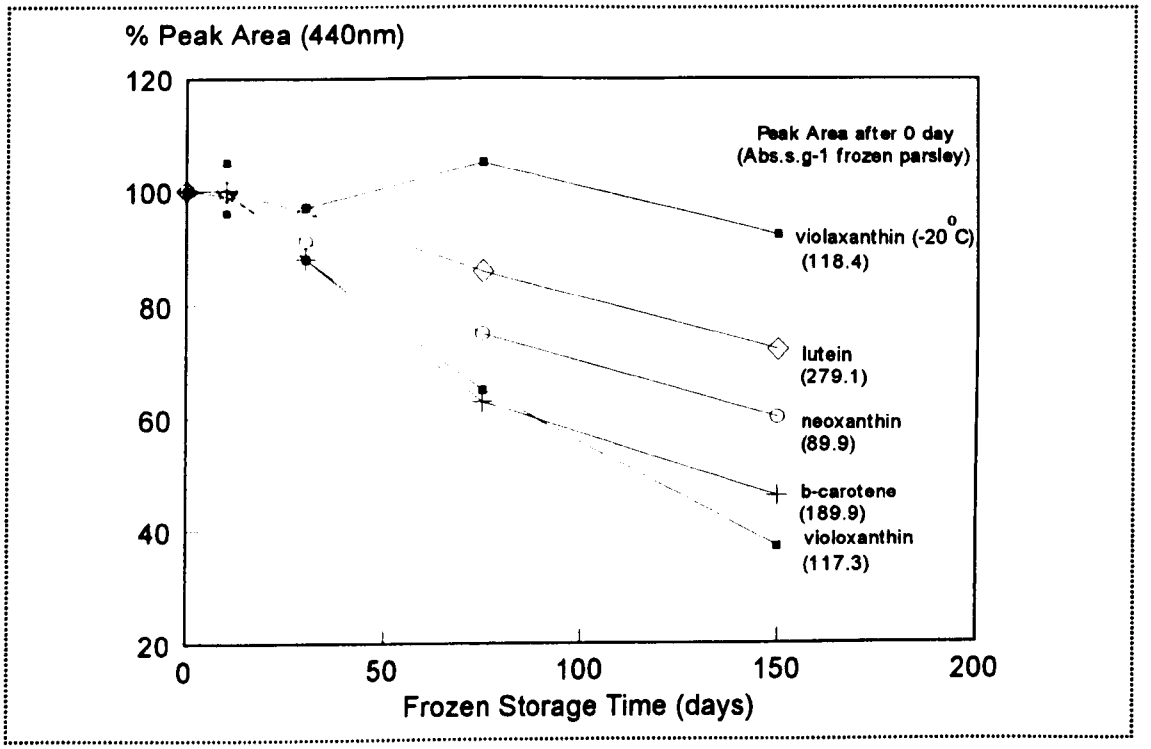


To summarize these data, 13^2-hydroxychlorophyll 'a', chlorophyllide 'a' and pheophytin 'a' are only minor products and appear to represent a relatively small fraction of the total level of chlorophyll 'a' degraded. In the data presented, the kinetics of chlorophyll loss do not follow that cited for pheophytin formation, during thermal treatment, nor is there an apparent stoichiometric relationship between chlorophyll 'a' and pheophytin 'a', suggesting that this conversion is not primarily responsible for the observed changes. Although 13^2-hydroxychlorophyll 'a' and chlorophyllide 'a' do not account for the stoichiometric loss of chlorophyll 'a' *per se*, they do display some transient character and therefore are potentially reactive species, acting either as a intermediate in the loss of chlorophyll or representing a minor side reaction. In Chapter 1.7, the hypothesis that peroxidase may be

operating by forming an oxidative couple with endogenous phenolic compounds, capable of the further oxidation of endogenous compounds would be consistent with these observations, however as stated above, chlorophyll 'a' is not a natural or direct substrate for peroxidase. Finally, as with the volatile compounds, it is believed that the bulk of the degradation products of chlorophyll 'a' are of higher molecular weight than the parent, as it would be expected to see lower molecular weight species within the largely overlapping analyte windows of the SNCVA/SNCNVA technique. An important exception to this situation, based on UV detection, as used in SNCNVA (photodiode array), would be if the absorbing elements of the chlorophyll molecule are lost as part of the degradation reaction. In this situation the molecule might remain undetected as it elutes from the HPLC column.

The effect of frozen storage on the carotenoid content of unblanched parsley, stored at -10°C, shows significant degradation of all the major carotenoids species under apparent zero order kinetics, (Figure 4.8, Table 4.2).

Figure 4.8 Effect of Frozen Storage (-10°C) on Carotenoid Level in Parsley.



Violaxanthin and β-carotene are the most reactive carotenoids however these carotenoids display relatively dissimilar chemical functionalities and polarities. Lutein is the least reactive carotenoid containing similar hydroxylation of the terminal C_6 rings to violaxanthin, however, with the exclusion of the epoxide groups. No obvious carotenoid degradation products are formed within any of the analyte windows suggesting again, the potential formation of higher MW products. As the carotenoids are known to be effective singlet oxygen quenchers, as well as potentially effective antioxidants via their reaction with peroxyl radicals (Gordon 1990) their depletion might be indicative of the type of processes operating in unblanched parsley.

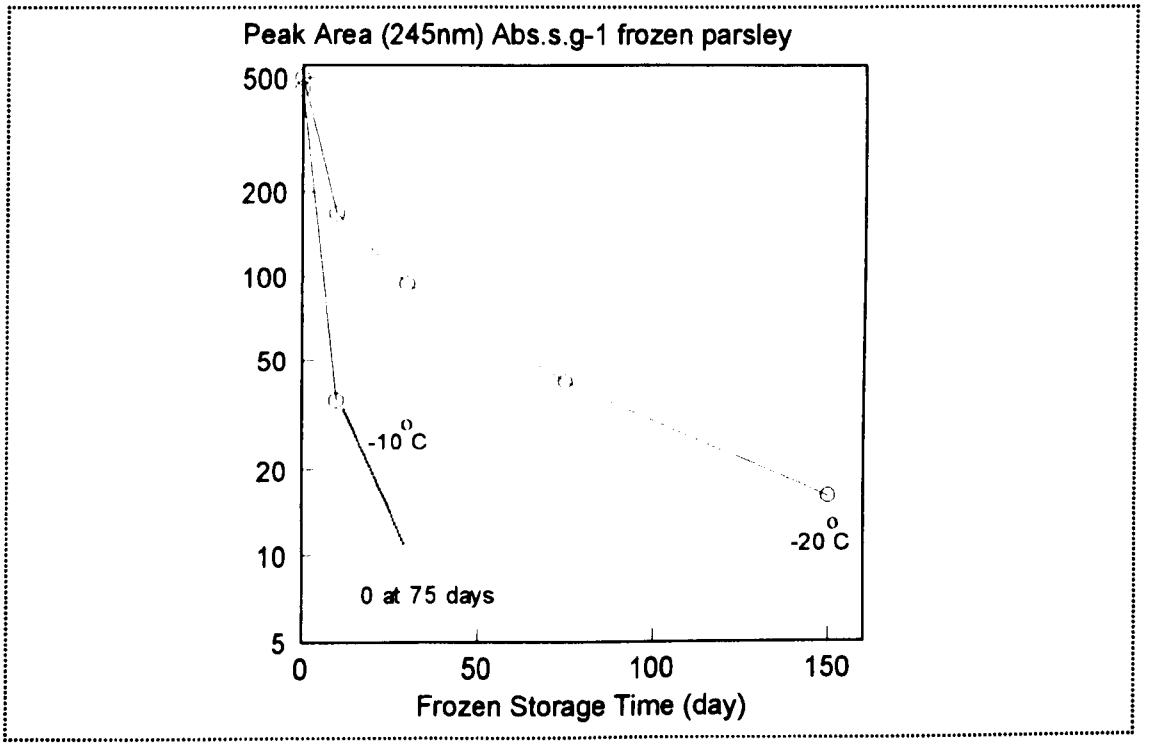
In comparing and contrasting literature on chlorophyll and carotenoid degradation with the data presented above, Amir-Shapira *et al.* (1987) studying detached parsley leaves senescing in the dark at ambient temperature, showed similar loss of chlorophyll 'a' with the accumulation of pheophytin 'a'. However, did not observe chlorophyllide 'a' formation. From their study, a comparable position for the degradation of carotenoid was also recorded, although only β-carotene was specifically identified in the HPLC chromatogram. Interestingly the behaviour of parsley under frozen storage appears to display a number of common features to ambient storage, which are relevant to the accelerated storage protocol implemented later in this thesis (Chapter 5). More recently, and within the time course of this study, Yamauchi *et al.* (1993) studied detached parsley leaves under modified atmospheres, recorded the transient appearance of both chlorophyllide 'a' and 13^2-hydroxychlorophyll 'a'. Additionally the group identified low levels of new pigments, suspected to be esterified xanthophylls resulting from the loss of the parent, however these species were not apparent in our chromatographic data.

4.2.4 Effect of Frozen Storage on Ascorbic Acid in Parsley.

The effect of frozen storage on ascorbic acid content showed a rapid degradation at -10°C with approximately 88% ascorbic acid degraded after 10 days (Figure 4.9). The rate of degradation at -20°C occurred more slowly and has been shown to follow apparent second order kinetics (Table 4.2). Ascorbic acid is a well known chemical antioxidant and can be consumed in this protective capacity and, as with the carotenoids, may be indicative

of the oxidative processes operating in unblanched parsley. As an alternative loss mechanism, plant tissue contains a specific enzyme, ascorbic acid oxidase. This enzyme is a peroxidase, and as with the many other peroxidases within plant tissue has a range of substrate specificities, and collectively allow for the additional potential loss of ascorbic acid (Dalton 1991, Takahama 1993). The significance of second order kinetics potentially allows information on the type of reaction, typically second order reactions can be related to polymerisation reactions (Pryor 1966), or to a complex reaction where two molecules of ascorbic acid are consumed in a cascade reaction.

Figure 4.9 Effect of Frozen Storage (-10°C) on Ascorbic Acid Level in Parsley.

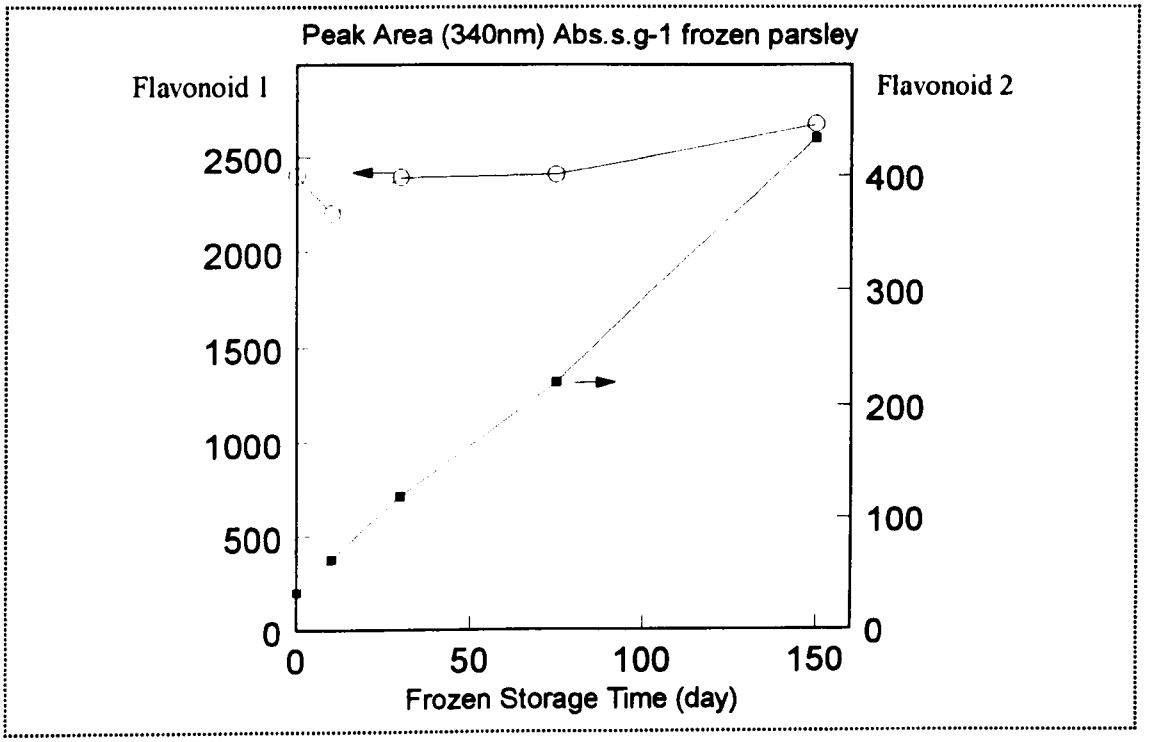


4.2.5 Effect of Frozen Storage on Flavonoids and Phenolics in Parsley.

The effect of frozen storage on the flavonoid composition of unblanched parsley stored at -10°C shows the major flavonoid ('Flavonoid 1'), tentatively assigned through UV spectroscopy and retention time, as apigenin-7-glucoside, remains unchanged over the storage period. A second flavonoid ('Flavonoid 2') with near identical spectra but eluting at a lower retention time, indicating an increased polarity (Chapter 3), is formed under apparent zero order kinetics (Figure 4.10, Table 4.2). 'Flavonoid 2' is a minor flavonoid

component, accounting for approximately 15% of the 'Flavonoid 1' component at 150 days and may be a polar derivative, in light of no other observed flavonoid changes. Flavonoids are natural substrates for peroxidase and have been implicated in a potential oxidative mechanism for the loss of chlorophyll in parsley, as reviewed in Chapter 1.

Figure 4.10 Effect of Frozen Storage (-10°C) on Flavonoid Level in Parsley.

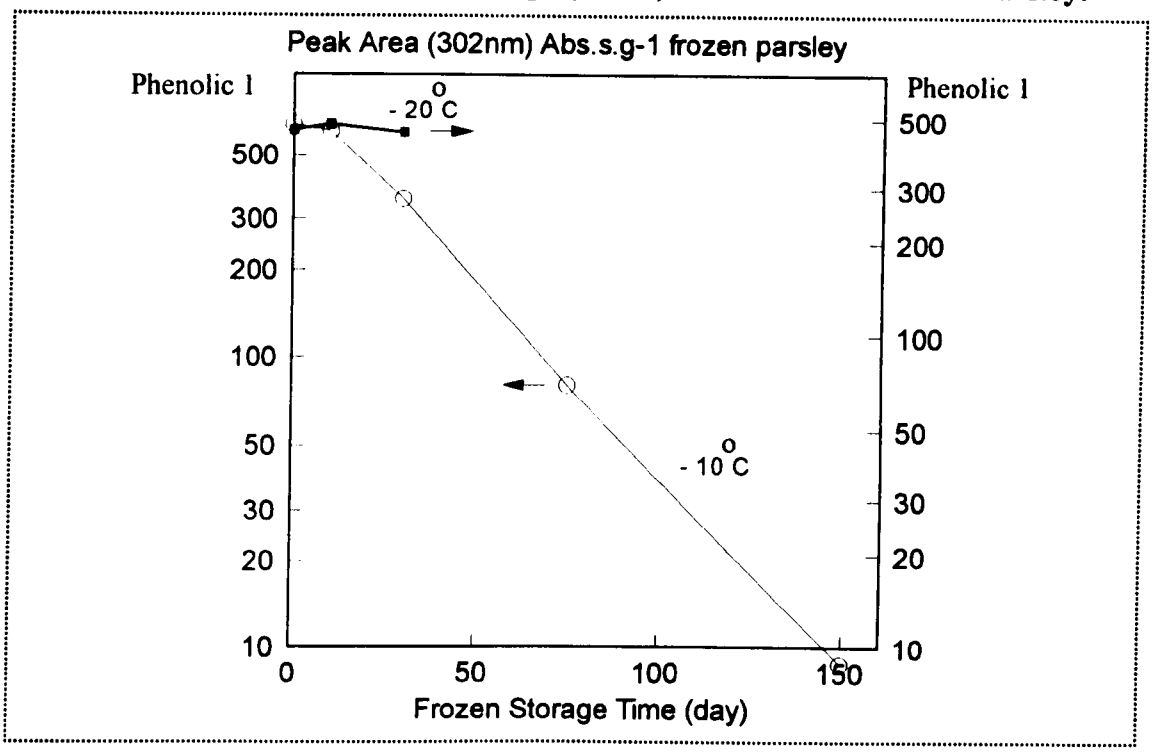


Interestingly, 'Flavonoid 2' correlates (linear; see Section 4.2.3) significantly with select analytes showing change on frozen storage and provides a linkage to these chemicals for further investigation. In particular, the correlation between the formation of 'Flavonoid 2' and the degradation of chlorophyll 'a' tentatively supports the involvement of the flavonoids (Table 4.3). Further experimentation on the chlorophyll/flavonoid system are discussed in Chapters 5 and 6.

The final class of analytes investigated are the phenolics, which like flavonoids are natural substrates for peroxidases and can be potentially viewed in the same context. The unknown phenolic, 'Phenolic 1', degraded on frozen storage under first order kinetics, and has been shown to correlate (linear; see Section 4.2.3) with 13^2-hydroxychlorophyll 'a' and p-cymenene as formation products, and with the degradation of menthatriene (Figure 4.11;

Table 4.2, 4.3). These relationships provide a linkage between these species and form the basis of further investigation, as discussed above for the flavonoids.

Figure 4.11 Effect of Frozen Storage (-10°C) on Phenolic Level in Parsley.



4.2.6 Qualitative Relationships between the Sensory and Chemical Status Of Parsley.

The aroma quality of parsley (unblanched) during frozen storage has been defined (Chapter 1.4, Figure 1.4, 1.6) and used to influence the technological processing under investigation within this thesis. On frozen storage, 'freshness' and 'grassy' aromas have been shown to decrease, whilst 'hay-like' aroma increases and predominate at longer storage times. Qualitatively, the decrease in 'freshness' correlates with the degradation of the monoterpenes. Myrcene (herbaceous, metallic) and menthatriene (terpeny) are two important compounds within this category which have been defined as organoleptically significant in fresh parsley, based on their 'flavour dilution' factor using AEDA analysis (Jung *et al.* 1992). Less information is available on the compounds which may be responsible for the decrease in 'grassy' aroma in frozen parsley, however hexanal (pungent, fatty-green, grassy) and trans-2-hexenal (pungent, green-fruity) are only generated when

freshly frozen parsley is thawed. Stored parsley appears to lose the capacity to generate these compounds. Qualitatively, the increase in 'hay-like' aroma correlates with the formation of p-cymenene. Organoleptic assessment of pure p-cymenene (diluted in propylene glycol; in house assessment) described the aroma as tar-like, old parsley, sweet, dry hay, and tentatively support this relationship. A similar assessment of pure menthatriene (in propylene glycol) described the aroma as harsher, 'not sweet', tar-terpeny. Old parsley and dry parsley were not perceived.

The colour quality of parsley (unblanched) on frozen storage has been shown to correlate with chlorophyll degradation (Philippon *et al.* 1986), and this relationship is qualitatively supported within this study.

4.3 Conclusions

Within this chapter, *in-vivo* analysis of a range of volatiles and non volatile analytes as a function of frozen storage has been performed using novel methodology designed as part of a unified analysis approach. These data have allowed the transient quality chemicals, and related species, within the tissue to be identified, along with various degradation products, and potentially forms a chemical basis for the quality deterioration phenomena in frozen parsley. From the chemical nature of the species involved, the basis for chemical/biochemical change has been considered. These reactions can be tentatively classified into four groups:-

(i) oxidation with respect to ascorbic acid loss and the formation of 13^2-hydroxy chlorophyll 'a', p-cymenene, hexanal and the tentatively assigned mentha-8-ene diepoxide.

(ii) polymerisation and the formation of high molecular weight species in relation to the depletion of menthatriene, chlorophyll 'a' and carotenoids.

(iii) protonation in the formation of pheophytin 'a' from chlorophyll 'a'.

(iv) enzymic hydrolysis of chlorophyll 'a' to chlorophyllide 'a'.

In the following chapters the aim is to investigate these reactions further with particular focus towards volatiles and chlorophyll pigments.

Chapter 5

Origins of Change of Flavour Quality Chemicals

5.0 Introduction

In the previous section, the endogenous chemicals affected by frozen storage were identified and classified by the type of the reaction associated with their loss. In this chapter the objective is to perform secondary *in-vivo* experimentation to establish the relative roles of chemical and enzymic processes, and to investigate further, the mechanisms of reaction. Initially, volatiles are considered using an accelerated storage protocol, to allow the assessment of several variables over a reduced time period. In these experiments, heat is used to deactivate enzymes and, oxygen control employed to investigate oxidative reactions. Finally, real time storage protocols are performed, using compable heat treatments, to confirm the accelerated storage data and extend the investigation to include non volatile species.

5.1 Experimental

5.1.1 The Effect of Frozen Storage (-10°C) on the Total Volatile Composition of Frozen Thawed Unblanched Parsley

SNCVA I analysis was performed on ~1.5g unblanched frozen parsley (1992 harvest) stored at -10°C for 0 day, 30 days, 75 days and 150 days. The protocol used was slightly modified, each sample was allowed to thaw from -65°C to 20°C, by immersion in a waterbath for a 5 minute period within the sealed vial. The sample were subsequently refrozen to -65°C and analysed using the standard SNCVA protocol.

Figure 5.1 displays the monoterpene region of the chromatograms for the frozen/thawed samples and compares them to the equivalent 'frozen only' samples (see Chapter 4).

5.1.2 The Effect of Accelerated Storage (20°C) on the Total Volatile Composition of Frozen Thawed Unblanched Parsley.

Frozen parsley (1992 Harvest) stored at -65°C was transferred (~ 1.5g) into 3 separate anti vortex vials and sealed at -65°C. The samples were allowed to thaw to room

temperature (20°C), by immersion in a waterbath, for 5 min, 4 h 40 min and 25 h before being refrozen to -65°C and analysed using the standard SNCVA protocol. Figure 5.2 'Series 2' displays the monoterpene region of the chromatograms.

5.1.3 The Effect of Open Blanching on the Total Volatile Composition of Frozen Thawed Parsley under Accelerated Storage.

Frozen parsley (1992 Harvest) stored at -65°C was transferred (~ 10g) to a stainless steel wire mesh blanching basket and immersed in boiling water (100°C) for 90 seconds. The basket was immediately dipped into iced distilled water and excess water removed from the tissue. The blanched material was transferred (approximately 1.5g) into 3 anti vortex extraction vials, sealed and held at room temperature (20°C) for 5 min, 4 h 40 min and 25 h before being refrozen to -65°C and analysed using the standard SNCVA protocol. Figure 5.2 'Series 3' displays the monoterpene region of the chromatograms.

5.1.4 The Effect of Sealed Blanching on the Total Volatile Composition of Frozen Thawed Parsley under Accelerated Storage.

Frozen parsley (1992 Harvest) stored at -65°C was transferred (~ 1.5g) to 3 separate anti vortex vials held at -65°C. The vials were sealed using a modified PTFE backed silicon rubber septa containing a glass capillary melting point tube sealed at one end and extending into the frozen parsley. A fine thermocouple was inserted into the capillary tube such that the metal junction was at the sealed extremity of the glass tube and within the frozen parsley (Figure 5.3). The vial arrangements were totally immersed into a large volume of boiling water (100°C) and held until the recorded temperature showed a value of >95°C for 2 min (total blanch time 7 mins; Figure 5.3). The sample vials were rapidly cooled, by immersing into an ice water bath, and held at to 20°C for 5 min, 4 h, 40 min and 25 h before being refrozen to -65°C and analysed using the standard SNCVA protocol. Figure 5.2 'Series 4' displays the monoterpene region of the chromatograms.

5.1.4.1 Determination of Peroxidase Activity in Parsley

Aliquots (5,10,15,20,25,50,75 µl) of a stock horseradish peroxidase solution (37.5 nM in

80mM pH 6.5 phosphate buffer) were assayed using the MBTH/DMBA method described in Chapter 2.1.3. A calibration curve was constructed plotting change in absorbance (mAbs/s) against peroxidase concentration (pM), (Figure 5.4/5.5).

5.1.4.2 The Effect of Sealed Blanching on the Peroxidase Activity in Parsley

Frozen parsley (1994 harvest) stored at -65°C was blanched as described in Section 5.1.4. Peroxidase activity for blanched parsley (5g), frozen parsley (5g) and fresh cabbage (5g) were determined using the extraction procedure/peroxidase assay described in Chapter 2.1.2/2.1.3 (Figure 5.5).

5.1.5 The Effect of Oxygen Removal from the Sample Headspace on the Total Volatile Composition of Frozen Thawed Parsley under Accelerated Storage.

Frozen parsley (1992 Harvest) stored at -65°C was transferred (~ 1.5g) to 3 separate anti vortex vials held at -65°C. Each vial was sealed with a PTFE/silicon septa containing two 0.7mm ID stainless steel tubes connected to a switched vacuum supply/helium supply and held at -65°C (Figure 5.6). A valve arrangement was utilized to cycle the headspace of the vial from atmospheric to 160mm Hg and back in 30 second and 60 second respectively, effectively replacing the evacuated air with 100% helium. The switching sequence was repeated 10 times to ensure effective oxygen removal (total time = 15 min at -65°C). Vial 1 was analysed directly using SNCVA I, vials 2 and 3 were allow to thaw to room temperature for 4 h 40 min and 25 h before being refrozen to -65°C and analysed using the standard SNCVA protocol. Figure 5.2 'Series 5' displays the monoterpene region of the chromatograms.

5.1.6 Effect of Sealed Blanching on the Volatile and Non-Volatile Composition of Frozen Parsley (-10°C) under Real Time Storage.

Frozen parsley (1994 Harvest) stored at -65°C was blanched as described in Section 5.1.4. After blanching, the sample vials were rapidly cooled (using crushed solid carbon dioxide), and held at -10°C for 0 days, 30 days, and 75 days. All samples, including a

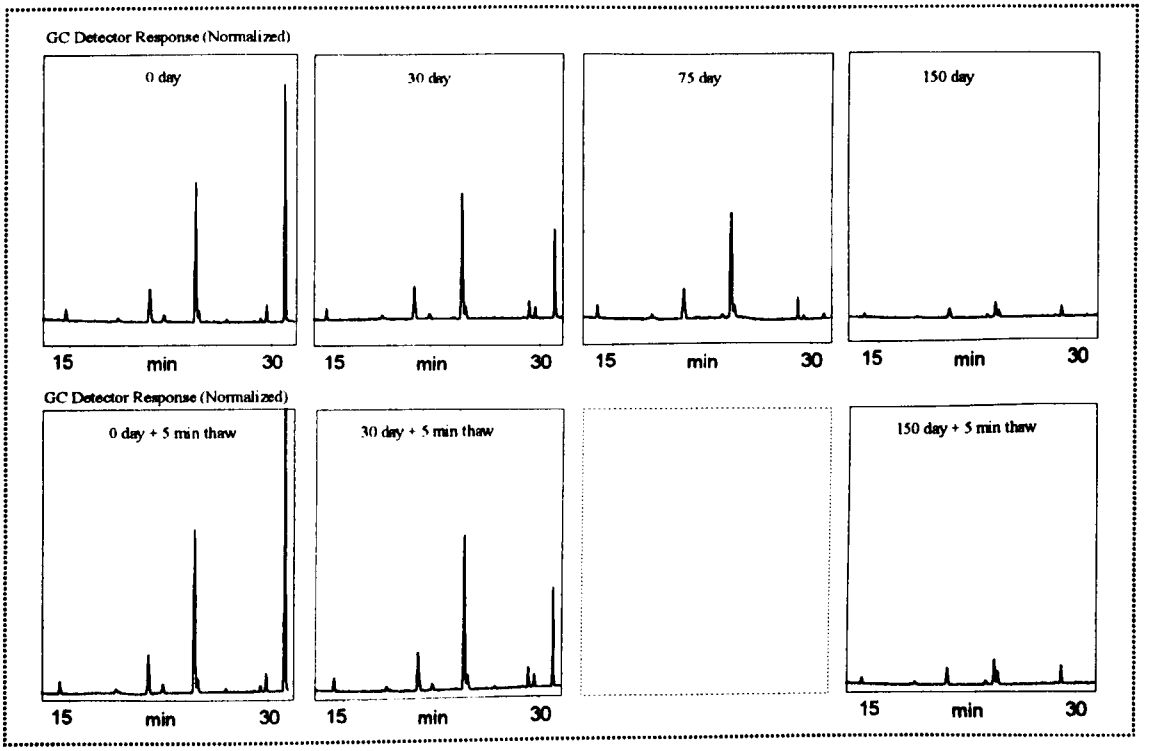
control sample taken before the blanch sequence (stored at -65°C), were analysed by SNCVA/SNCNVA analysis. Figures 5.8 to 5.15 compare the chromatographic peak area, as a function of storage time, for the above samples against the associated unblanched samples recorded in Chapter 4.

5.2 Results and Discussions

5.2.1 Effect of Frozen Storage on the Volatiles of Frozen Thawed Parsley.

The effect of thawing frozen parsley (5 minute thaw period) appears to result in little change to the main gravimetric components, over the comparable frozen samples (Figure 5.1). The chromatographic data shown represents the monoterpene region of the chromatogram, identified to contain the compounds sensitive to degradation on frozen storage (Figure 4.1).

Figure 5.1 Effect of Frozen Storage on the Volatile Composition of Frozen (Upper) and Thawed Unblanched(Lower) Parsely.



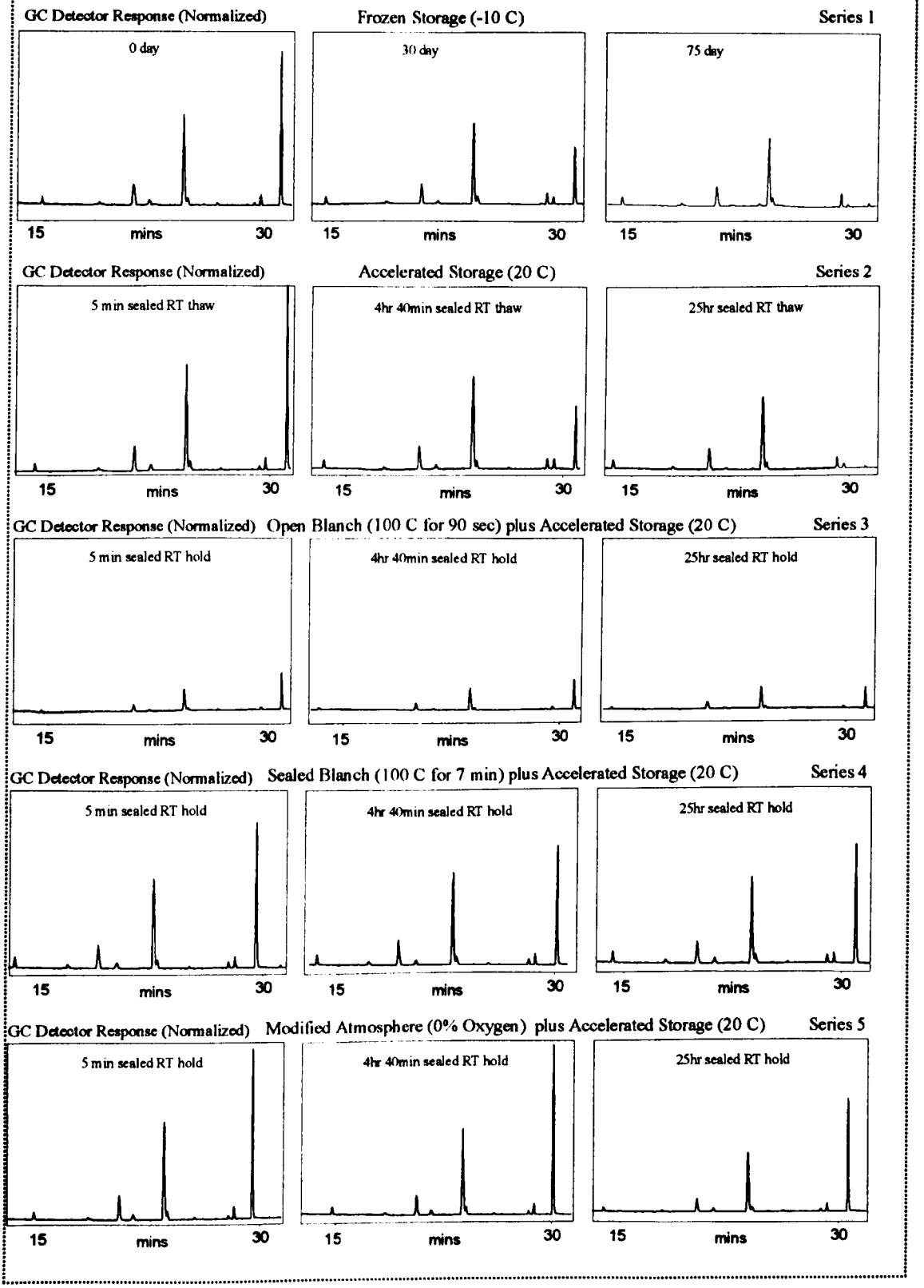
The ordinate axis (y-axis) records the absolute GC detector response, normalized for the weight of parsley analysed (Chapter 3.3.2.1), and is comparable between all chromatograms. Minor C_6 aldehydes are observed as discussed in Chapter 4 (Figure 4.5).

These findings are important in establishing the contribution of thawing to the proposed accelerated storage protocol (20°C) in which a thaw stage is inherent.

5.2.2 Effect of Accelerated Storage on the Volatiles of Frozen Thawed Parsley.

In preliminary experiments, using a trial and error approach, it was found that a 5 minute, 4 hour 40 minute and 25 hour extended thaw at 20°C was equivalent in volatile composition terms to frozen storage at -10°C for 0 day, 30 days and 75 days respectively (Figure 5.2 'Series 1 and 2'). From these data it can be tentatively proposed that the reactions operative at -10°C, and responsible for volatile loss, are also operative at 20°C. In this situation the accelerated storage protocol would represent frozen storage and would potentially allow a series of experimental conditions to be assessed over a 25 h period rather than 75 or 150 days. Further tentative evidence to support this proposal was highlighted in Chapter 4.2.3 discussing the degradation of chlorophyll and carotenoids. Evidence for a single process occurring at the two temperatures of investigation can also be potentially obtained by considering the chemical kinetics as a function of temperature change, namely Arrhenius behaviour. To date the chemical kinetics of menthatriene loss at -10°C has been shown to be first order with respect to menthatriene. From the kinetics plot (Figure 4.3) the rate constant for the reaction can be obtained. Determination of the rate constant at a range of temperatures spanning the ice transition temperature would allow a standard Arrhenius plot to be constructed by plotting ln (rate constant, k) vs 1/temperature. A straight line would indicate Arrhenius behaviour and suggest a conformity within the reaction over the temperature range investigated. Additionally the activation energy (Ea) of the reaction can be obtained from the slope of the plot, and offers an indication of whether the reaction is chemical or enzymic in nature. Typically chemical reactions have activation energies greater than 63 kJ mol^{-1} whereas many enzymic reactions range from ~1 to 33 kJ mol^{-1} (Conn *et al.* 1976, Atkins 1982). From the data generated in this section only two temperatures have been evaluated which is clearly insufficient to establish the linearity of the Arrhenius plot and could only tentatively be used to estimate an activation energy for the reaction. Finally, the validity of data generated under an accelerated regime must be supported from real time experiments (see Section 5.2.6).

Figure 5.2 Effect of Blanching and Modified Atmosphere on the Volatile Composition of Parsley Under Accelerated Storage Conditions.



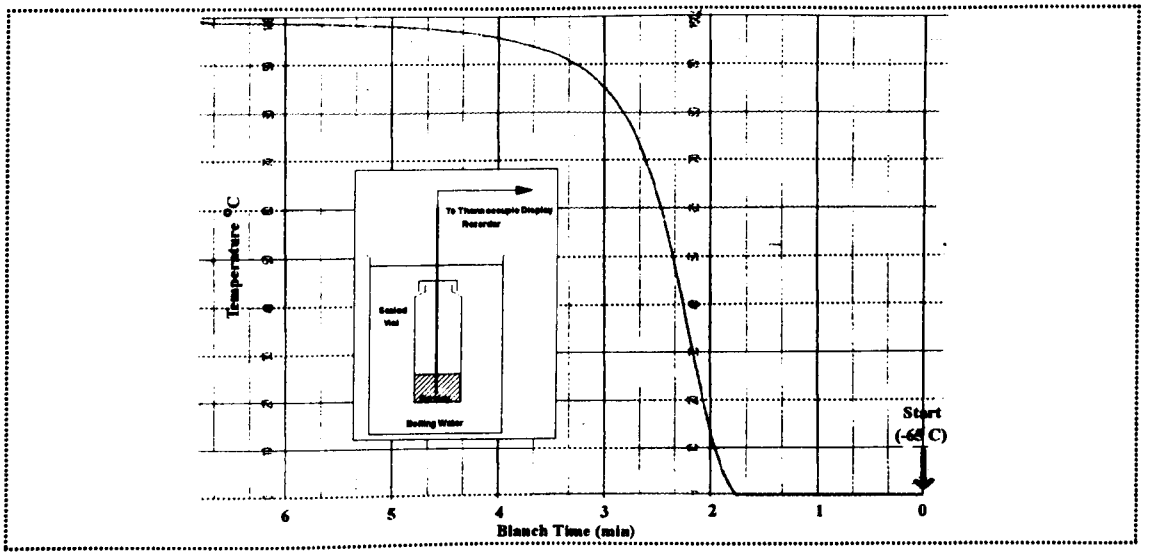
5.2.3 Effect of Open Blanching on the Volatiles of Frozen Thawed Parsley.

Commercial processing of parsley typically excludes a blanching stage due to the extensive loss of aroma. Commercial immersion blanching plants generally utilize water tanks open to the atmosphere. This arrangement has been used to design a relevant laboratory sequence, termed 'open blanching' (Section 5.1.3), to evaluate the effect of a typical blanching protocol. The effect of 'open blanching' on the volatile composition of parsley shows an extensive reduction in the overall level of volatiles, although the volatile profile is largely retained over the accelerated storage period (Figure 5.2 'Series 3'). Increased stability is also shown by the previously changing monoterpenes (Figure 5.2 'Series 1 and 2'), suggesting a possible contribution from an enzymic process. Within this sequence, the mechanisms of volatile loss will clearly involve evaporation and partitioning into the blanch water, however their relative contribution and the role of chemical processes, at these elevated temperatures is unknown at this stage.

5.2.4 Effect of Sealed Blanching on the Volatiles of Frozen Thawed Parsley.

Based on the extensive loss of volatiles during 'open blanching', a 'sealed blanch' protocol was designed to control the losses due to evaporation and partitioning into the blanch water, and therefore to identify the contribution made by other processes, for example due to chemical change (Figure 5.3).

Figure 5.3 Temperature Profile During 'Sealed Blanching'.



The effect of 'sealed blanching' on the volatile composition of parsley shows the retention of volatiles with little or no loss over the accelearted storage period (Figure 5.2 'Series 4'), and contrasts significantly with the extensive volatile loss from unblanched parsley (Figure 5.2 'Series 2'). These data suggest that an enzymic process is responsible for the volatile loss during accelerated storage and that chemical degradation does not contribute significantly, even though the heating regime is relatively severe (100°C 7 min). Based on the earlier proposition that the accelerated storage protocol is representative of tissue under frozen storage, these conclusions can be tentatively drawn for frozen parsley. Although enzymes have been implicated in the loss of menthatriene, it is unclear, based on the chemical functionality of the molecule and the known substrates of the oxidoreductase enzymes (lipoxygenase, peroxidase, polyphenol oxidase), which enzyme might be responsible.

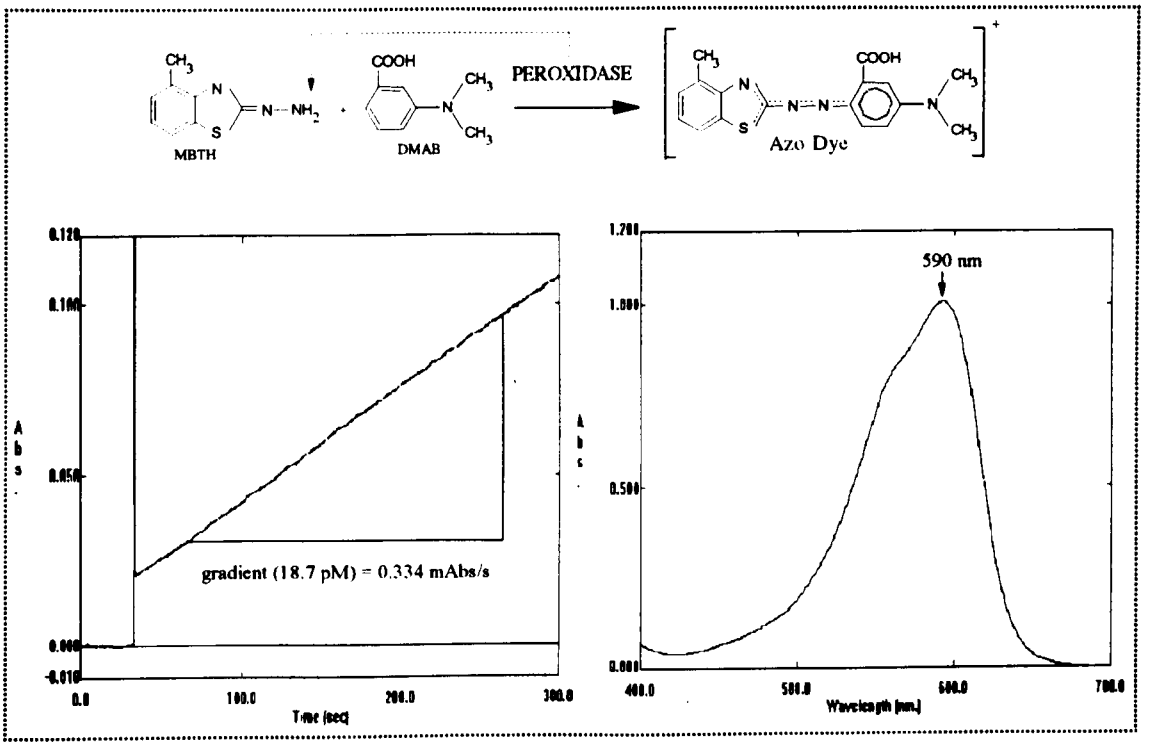
Evaporative loss and losses due to the partitioning of volatiles into the blanch water, predominant in open blanching, are extensively controlled using the sealed blanch protocol.

5.2.4.1 Determination of Peroxidase Activity in Parsley

Peroxidase activity is often utilized as a indicator for blanching, due to its relatively high heat tolerance (Williams *et al.* 1986). This enzyme has also been implicated in the loss of colour during the frozen storage of parsley (Chapter 1) and therefore would appear to be a relevant indicator within this study. The primary consideration in the selection of a suitable peroxidase assay is the safety, sensitivity and versatility of the method. Typical colorimetric assays utilize a colourless substrate which is oxidized by the enzyme. The oxidized molecule subsequently polymerizes or couples with a secondary reagent to form a stable chromophore (Maehly *et al.* 1954). Ngo *et al.* (1980) described a coupled reaction using 3-methyl-2-benzothiazolinone hydrazone (MBTH), as the initial substrate, coupled to 3-(dimethylamino) benzoic acid (DMAB) to produce an indamine dye (Figure 5.4). Initial characterisation experiments showed a linear rate of colour formation at 590 nm using the protocol described in Chapter 2.1.3 (Figure 5.4). In comparison with a standard guaiacol assay the MBTH/DMAB method successfully measured standard peroxidase solutions of the order 10-20 times less concentrated (data not given). Sensitivity is

particularly relevant to blanched tissues where low levels of residual peroxidase activity often lead to a degree of activity regeneration on storage. Based on the sensitivity and the reported safety of the reagents , the MBTH/DMAB assay was utilized in this study.

Figure 5.4 MBTH/DMAB Peroxidase Activity Assay (Ngo *et al.* 1980).



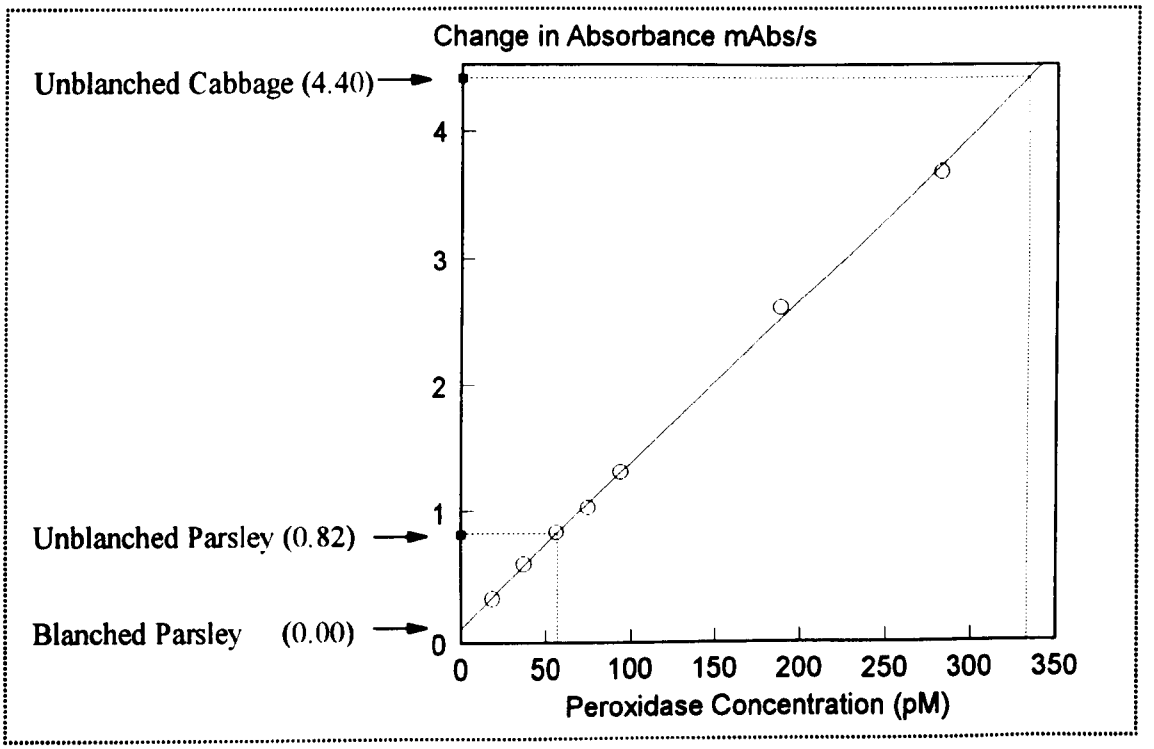
The MBTH/DMAB assay was calibrated using a series of standard peroxidase solutions (18.75 pM - 281.25 pM), calculated using a molecular weight for peroxidase of 44,000 (Sigma data sheet; Chapter 2.0). The rate of absorbance change (mAbs/s) was plotted against peroxidase concentration (pM) and shown to follow a linear relationship (R^2=0.9985; n=7; Figure 5.5). The gradient of the calibration graph obtained in this study showed good agreement with the data published by Ngo *et al.* (1980), 0.019 mAbs/s/pM and 0.013 mAbs/s/pM respectively.

5.2.4.2 The Effect of Sealed Blanching on the Peroxidase Activity in Parsley.

Preliminary experimentation to determine a suitable extraction protocol for peroxidase, from parsley, was performed. In this investigation, peroxidases were considered to be present in four different forms, namely soluble, ionically bound, membrane bound

(hydrophobic bonding) and covalently bound to the cell wall. Extraction conditions utilized combinations of sodium chloride (0.5 M, 1.0 M - ionic decoupling), Triton X-100 (1%w/v - hydrophobic decoupling) and cellulase/pectinase/hemicellulase mixture (24 h/25°C incubation- cell wall digestion). Increased sodium chloride level had the greatest effect on peroxidase activity, whereas Triton X-100 and the cellulase mixture only showed minor changes (data not shown). From these investigations, a phosphate buffer (80 mM pH 6.5) containing 1.0 M sodium chloride was shown to be most effective extractant combination and has been utilized in this study (Chapter 2.1.2).

Figure 5.5 Effect of 'Sealed Blanching' on Peroxidase Activity.

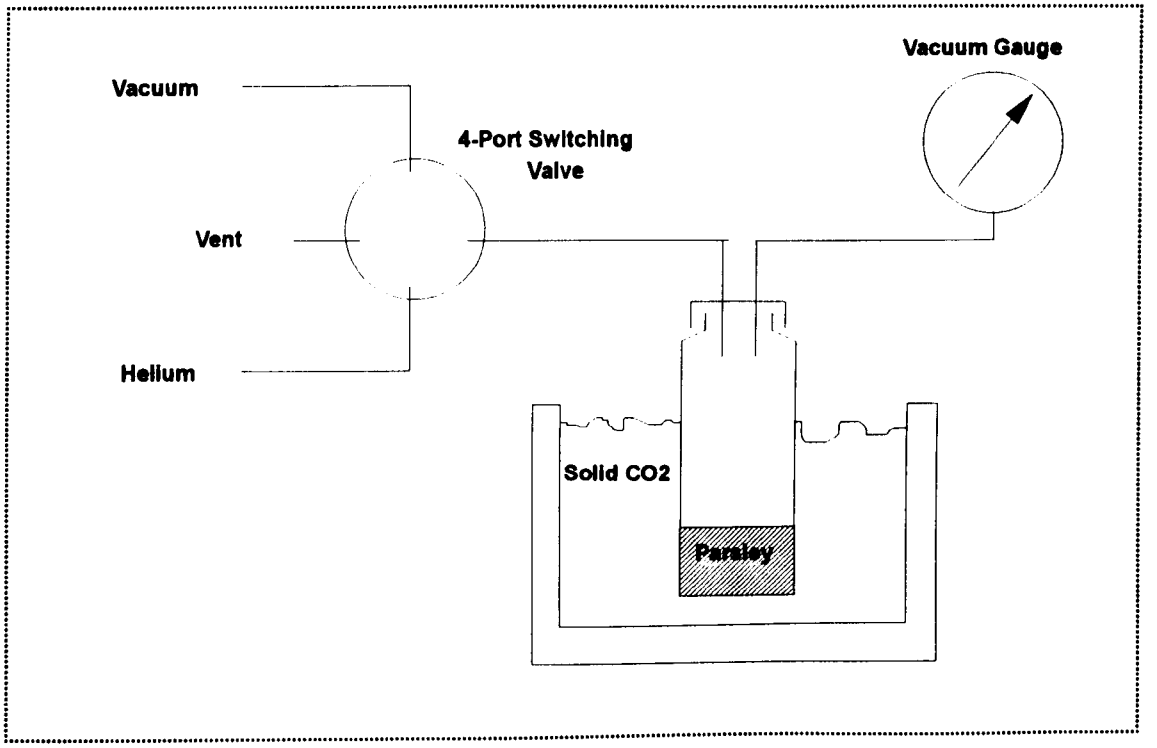


The peroxidase activities for unblanched parsley (frozen) and unblanched cabbage (fresh) were determined (Figure 5.5). The relative activity of cabbage (4.40 mAbs/s) was shown to be approximately 5 times greater than parsley (0.82 mAbs/s) and appears to be consistent with data reported in literature (Baardseth *et al.* 1987). Blanched parsley (frozen) was similarly assayed and shown to contain no peroxidase activity (0.00 mAbs/s). These data suggest the thermal treatment used in the 'sealed blanch' protocol was sufficient to inactivate peroxidase.

5.2.5 The Effect of Oxygen Removal on the Volatiles of Frozen Thawed Parsley.

Oxidation products have been experimentally shown to form during frozen storage whilst many endogenous compounds degrade over a comparable time period (Chapter 4). The effect of removing oxygen from the headspace over unblanched parsley on the volatile composition, showed the extensive retention of monoterpene volatiles over the accelerated storage period (Figure 5.6; Figure 5.2 'Series 5').

Figure 5.6 Headspace Modification Profile.



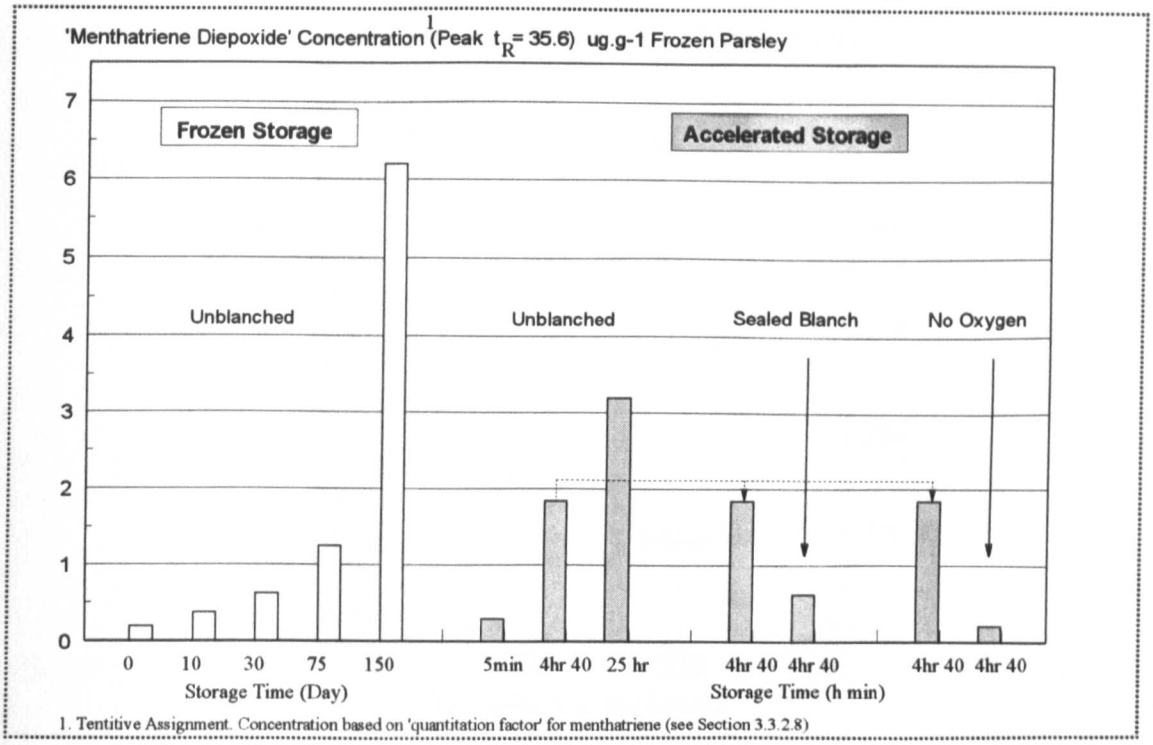
These volatiles, particularly menthatriene, have been shown to degrade during a similar storage protocol in the presensce of oxygen and establish the involvement of oxygen in a potentially enzymic based reaction. The oxidoreductase class of enzymes may be considered as the most likely causative enzymes, however it is not immediately apparent which enzyme might be responsible, based on known substrates and oxygen requirements. For example, the oxygenases, lipoxygenase and polyphenol oxidase known to consume oxygen, would be expected to be operative against a 1, 4 pentadiene moiety and a mono/ortho di phenol respectively. Equally unclear is the role of peroxidase requiring hydrogen peroxide and an 'enolic' substrate but without the requirement for oxygen.

Initially, lipoxygenase might warrant further consideration due to the presence of a diene moiety in the menthatriene molecule, however the double bond configuration is 1, 3 and isomerization would be necessary to meet the enzyme's 1, 4 requirement, assuming a 1, 4 arrangement in a cylic molecule would still be a substrate for lipoxygenase. As an alternative mode of action, the oxidoreductase enzymes have also been shown to participate in coupled or co-oxidation reactions where a natural substrate of the enzyme is oxidised and in turn co-oxidises a third party species, for example the co-oxidation of carotenoids by lipoxygenase in bread making. Under such a mode of action, the criteria of considering quality related compounds (eg menthatriene, chlorophyll) as known substrates for a particular oxidoreductase enzyme is not appropriate, rather consideration should be given to all endogenous species which are potential natural substrates of the oxidoreductases and, in turn capable of oxidizing the principal quality chemicals. Typically, these compounds are non-volatile (Chapter 1) and are the focus for investigation in the following sections (see Section 5.2.6, Chapter 6).

In Chapter 4 (4.2.1), the work of Hartmann (1985) was discussed in relation to the formation of a 1,2-3,4-diepoxide of menthatriene on the frozen storage of parsley and was compared and contrasted with the data obtained in this study. In the study he suggested that photoxidation was responsible for the degradation of menthatriene via the 1,4 addition of singlet oxygen to produce the 1,4 endoperoxide and the subsequent rearrangement to the 1,2-3,4-diepoxide. As an alternative degradation mechanism, Hartmann investigated the co-oxidation role of lipoxygenase/fatty acid. In model experiments, using parsley extracts with linoleic acid and soya lipoxygenase he observed the degradation of monoterpenes, particularly menthatriene (100% degradation). However he did not record any increase in the 1,2,-3,4-diepoxide as expected from their frozen storage data. The presence of the 1,2-3,4-diepoxide of menthatriene in our data was considered in Chapter 4 (4.2.1). A minor peak (t_R=35.6 min), consistent with the retention time (t_R) from Hartmann's data was identified and shown to form during frozen storage. Further investigation of this peak (t_R=35.6 min), as a function of thermal treatment and oxygen control, performed in this section, show that it is formed under accelerated storage and that formation is inhibited by blanching (enzyme deactivation) and the removal of oxygen (Figure 5.7). These data are consistent with the degradation behaviour of menthatriene and tentatively suggest that the 1,2-3,4-diepoxide (peak at t_R=35.6 min) may be a minor

degradation product of the parent. The disparity between the levels of the 1,2-3,4-diepoxide observed to form in Hartmann's work (13 mg/kg foliage ≡ ~25% menthatriene loss), and the lower levels tentatively found in this study (1.25 $\mu g.g^{-1}$ frozen parsley ≡ ~0.05% menthatriene loss, after 75 day at -10°C; Table 4.1; Figure 5.7), as well as the basis of degradation are discussed further in Chapter 6 (6.2.2).

Figure 5.7 Effect of Blanching and Modified Atmosphere on 'Menthatriene Diepoxide' Under Frozen and Accelerated Storage.



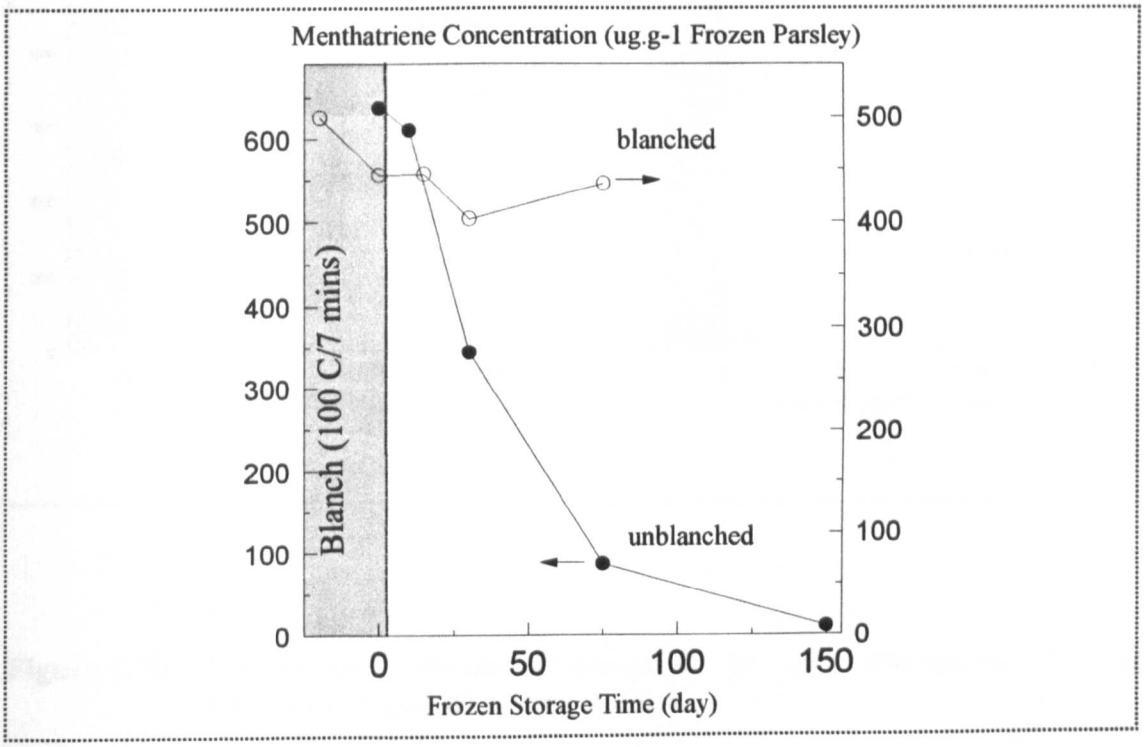
1. Tentitive Assignment. Concentration based on 'quantitation factor' for menthatriene (see Section 3.3.2.8)

5.2.6 Effect of Sealed Blanching on the Volatile and Non-Volatile Composition of Frozen Parsley Under Real Time Storage.

In the previous section, parsley has been tentatively shown to contain an enzymic based oxidative system capable of degrading endogenous volatiles. In this section, a comparable thermal treatment, shown to inactivate peroxidase and prevent volatile degradation, is similarly applied to identify non-volatile compounds which may be affected by or associated to this oxidative system. For example, chlorophyll is investigated as a quality chemical related to colour, whereas the carotenoids, ascorbic acid and the flavonoids are targetted as indicators of oxidative reactions (Chapter 1).

The effect of frozen storage under real time conditions, on the total volatile composition of 'sealed blanched' parsley shows the extensive retention of volatiles. Figure 5.8 compares the loss of menthatriene in unblanched parsley with the retention of menthatriene in 'sealed blanched' parsley and shows good agreement with the observations generated using an accelerated storage protocol.

Figure 5.8 Effect of 'Sealed Blanching'/Frozen Storage (-10°C) on Menthatriene in Parsley.



The effect of 'sealed blanching' on the chlorophyll 'a' level in parsley shows a large initial reduction followed by a less rapid loss on frozen storage (-10°C) at a rate similar to that displayed by unblanched parsley (Figure 5.9). Associated to the loss of chlorophyll 'a' is the linearly correlated formation of pheophytin 'a' ($R^2 = 0.9588$), with an approximate 92% conversion between the species, as calculated form the gradient of the correlation plot and based on quantitation of chlorophyll 'a' and pheophytin 'a' at wavelength 430nm and 410nm respectively (Figure 5.10). In contrast to the conversion in unblanched parsley (determined as 24%; Chapter 4), for blanched parsley pheophytin 'a' formation, through the acid mediated protonation of the porphyrin ring) extensively accounts for the loss of chlorophyll 'a'.

Figure 5.9 Effect of 'Sealed Blanching'/Frozen Storage (-10°C) on Chlorophyll 'a' and Pheophytin 'a' in Parsley.

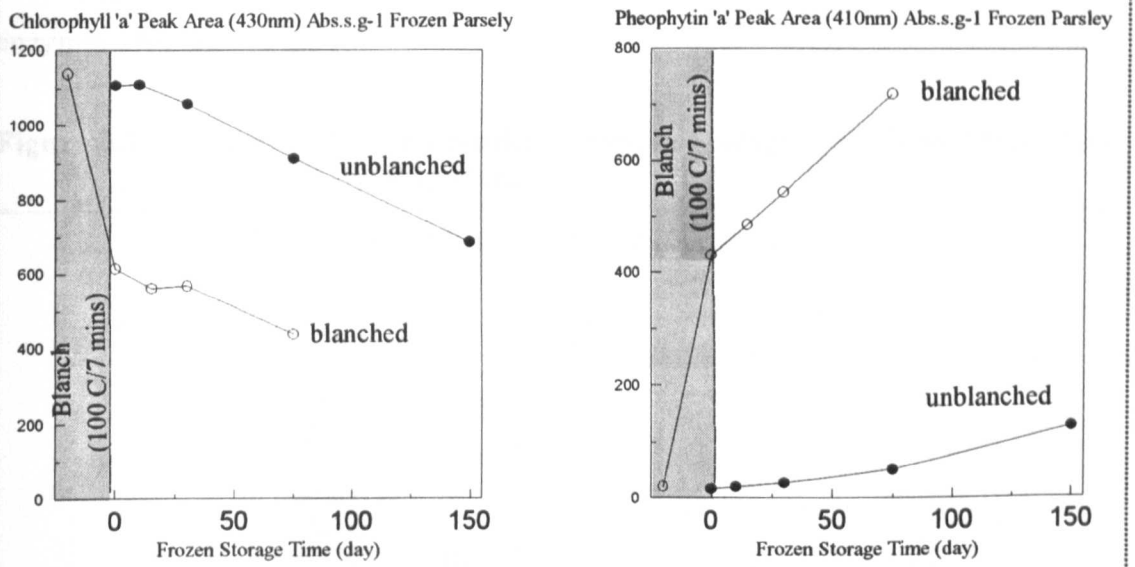
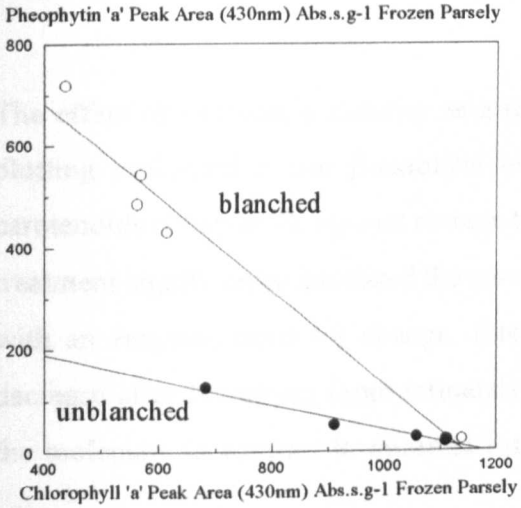


Figure 5.10 Correlation between Chlorophyll 'a' and Pheophytin 'a' in Unblanched and Blanched Parsley.

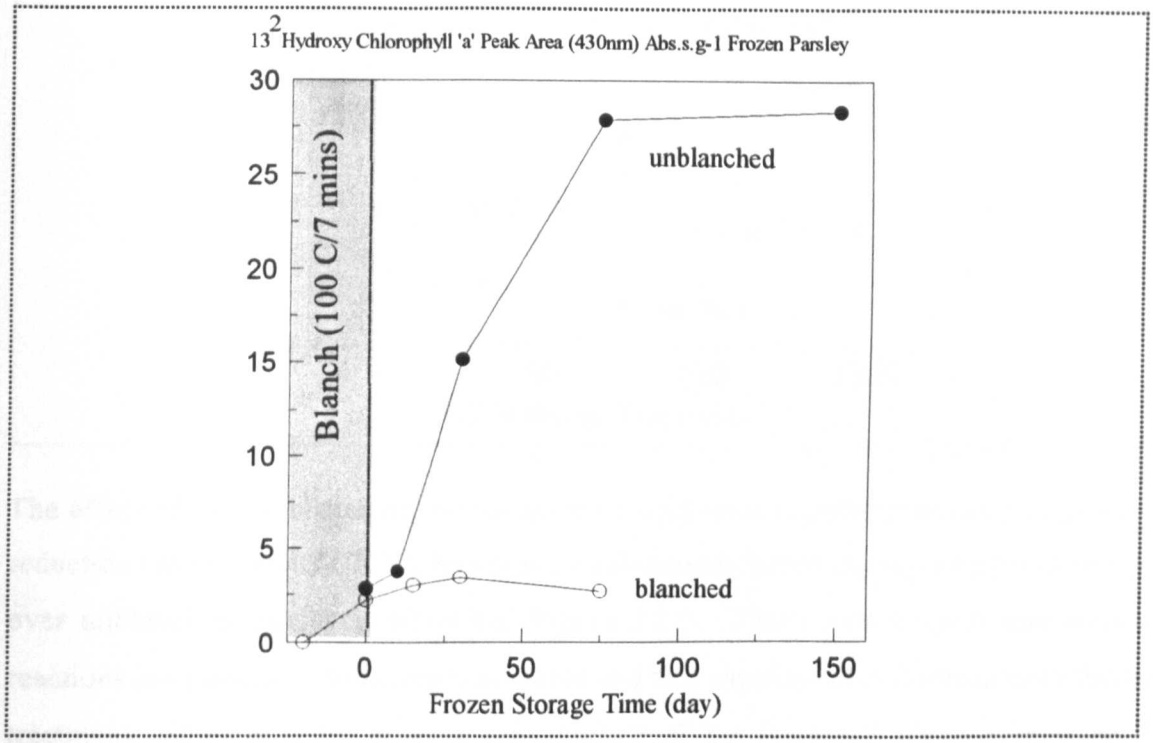


Regression Output: Unblanched Parsley	
Constant	288.13
Std Err of Y Est	8.8093
R Squared	0.9717
No. of Observations	5
Degrees of Freedom	3
'X Coefficient (Value x 100 = % Conversion)	-0.2456
Std Err of Coef.	0.0242

Regression Output: Blanched Parsley	
Constant	1051.68
Std Err of Y Est	60.3316
R Squared	0.9588
No. of Observations	5
Degrees of Freedom	3
X Coefficient (Value x 100 = % Conversion)	-0.9221
Std Err of Coef.	0.1103

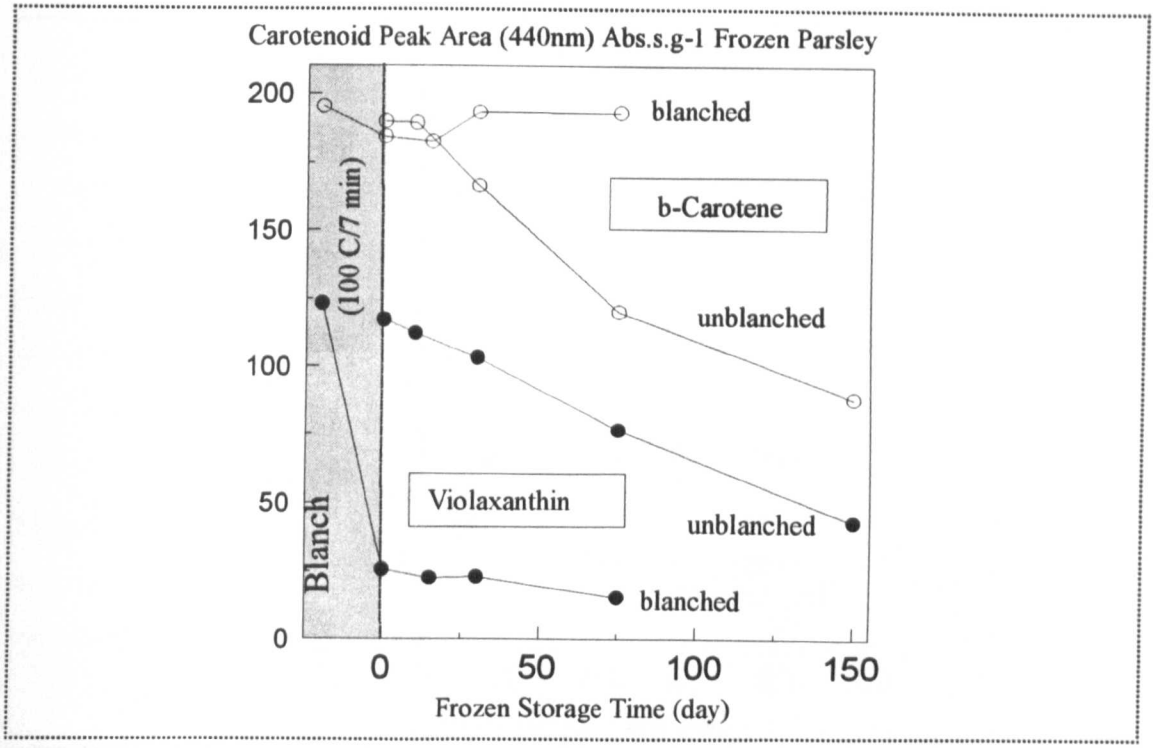
Additional support for the absence of an additional mechanism for chlorophyll loss, as identified in unblanched parsley, is the significantly reduced levels of 13^2-hydroxy chlorophyll 'a', an oxidized form of chlorophyll 'a' (Figure 5.11). These data tentatively suggest that thermal treatment extensively prevents the formation of 13^2-hydroxy chlorophyll 'a' and that the oxidation of chlorophyll to this product is associated with an enzymic process.

Figure 5.11 Effect of 'Sealed Blanching'/Frozen Storage (-10°C) on 13^2-Hydroxy Chlorophyll 'a' in Parsley.



The effect of carotenoid stability as a function of a 'sealed blanch' protocol is shown by plotting violaxanthin and β-carotene levels, previously identified as the most reactive carotenoids (Chapter 4), against storage time at -10°C (Figure 5.12). The effect of thermal treatment significantly increased the storage stability for both carotenoids and is consistent with an enzymic basis for change. Interestingly, violaxanthin displays a marked initial decrease after blanching (approximately 75%), due perhaps to the thermal instability of the molecule, in contrast to minimal loss for β-carotene.

Figure 5.12 Effect of 'Sealed Blanching'/Frozen Storage (-10°C) on Carotenoids in Parsley.



The effect of 'sealed blanching' on the ascorbic acid level in parsley shows a large initial reduction (approximately 70%), however on subsequent frozen storage, improved stability over unblanched parsley is observed (Figure 5.13). These data suggest that enzymic reactions are associated to ascorbic acid loss and that ascorbic acid is sensitive to thermal treatment, either directly, or as a consequence of the increased reaction rates of the degradation reactions associated to elevated temperatures (see Chapter 4.2.4).

The effect of 'sealed blanching' on the formation of 'Flavonoid 2', proposed to be a derivative of the parent flavone (apigenin-7-glucoside, 'Flavonoid 1', Chapter 4.2.5), shows a large initial increase followed by degradation on frozen storage (Figure 5.14). These data suggest the formation reaction is initially accelerated on thermal treatment and that a degradation reaction occurs on subsequent frozen storage. In previous experiments, parsley has been shown to contain an enzymic based oxidative system and peroxidase has been cited as a possible canditate, operating in a co-oxidative capacity involving the flavonoid, apigenin (Chapter 4).

Figure 5.13 Effect of 'Sealed Blanching'/Frozen Storage (-10°C) on Ascorbic Acid in Parsley.

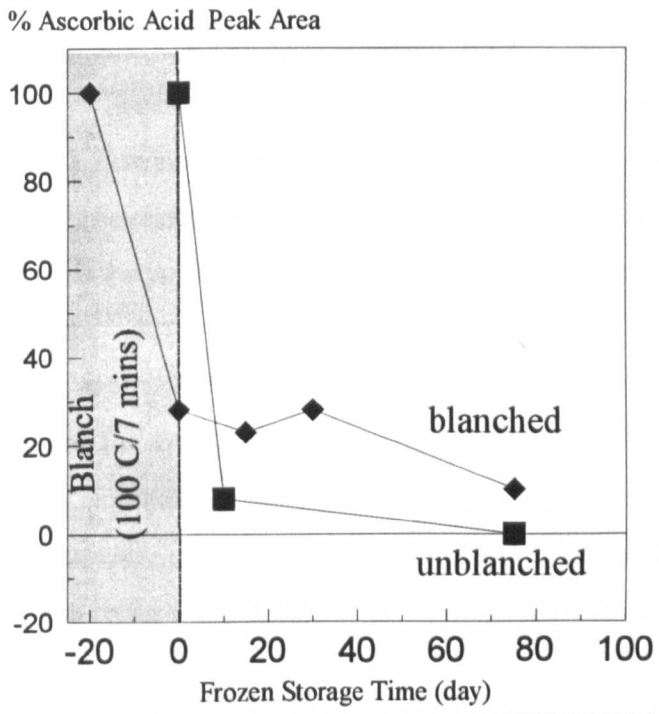
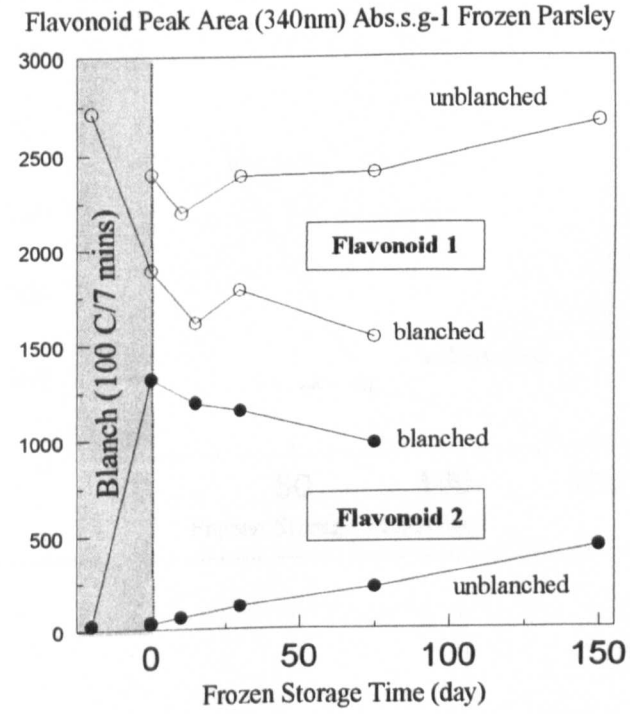
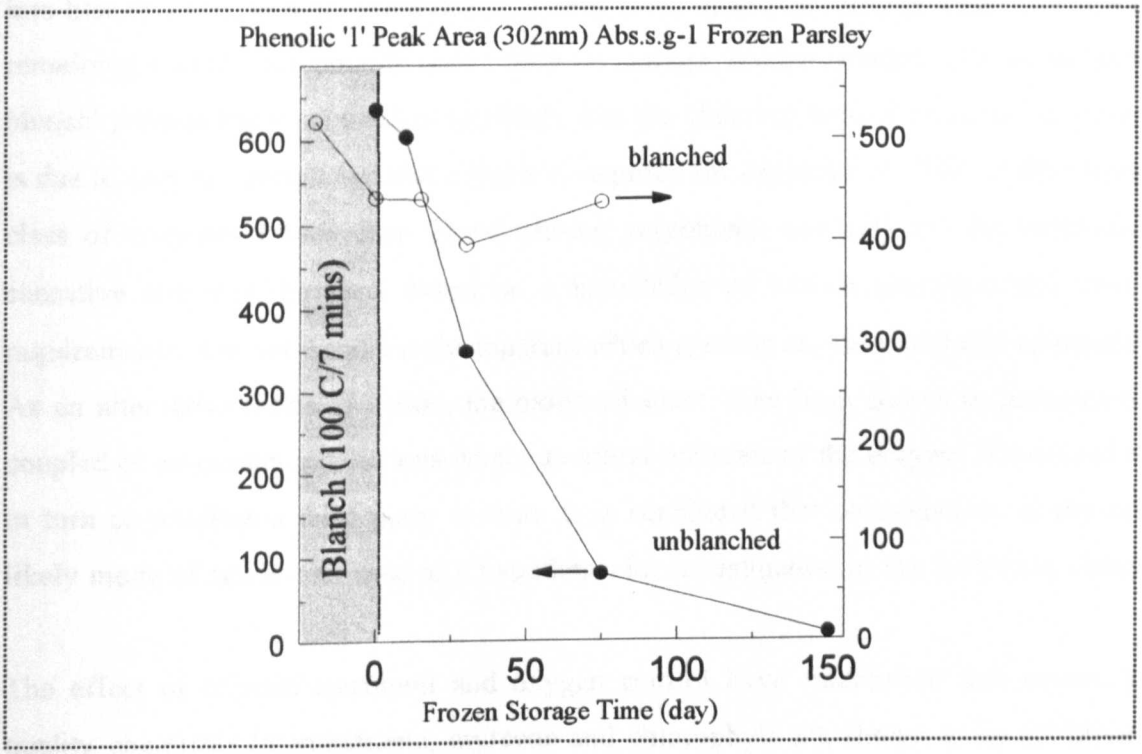


Figure 5.14 Effect of 'Sealed Blanching'/Frozen Storage (-10°C) on Flavonoids in Parsley.



These data can be tentatively rationalized by considering 'Flavonoid 1' (apigenin-7-glucoside) as to be a substrate for peroxidase and that 'Flavonoid 2' is a polar oxidation product produced by the action of peroxidase. On thermal treatment the enzyme reaction, and thus 'Flavonoid 2', increases until thermally inactivated, after which the unstable oxidized flavonoid degrades on subsequent frozen storage. In comparing the data with unblanched parsley, we observe the oxidized product to be formed under zero order kinetics (Chapter 4.2.5), however in the light of the above information, this situation may reflect the combined processes of formation and loss, and that thermal treatment deactivates the enzymes responsible for formation. In this earlier Chapter (4.2.5) little evidence to support the conversion of 'Flavonoid 1' to 'Flavonoid 2', based on the gravimetric status was available, due to the low level of 'Flavonoid 2' formed, although comparable UV spectra for the two compounds were obtained (Figure 3.18). However within these blanching experiments 'Flavonoid 1' is shown to display a significant initial decrease, on heat treatment, concurrent with a similar increase in 'Flavonoid 2', and tentatively supports the relationship between these species, presented above.

Figure 5.15 Effect of 'Sealed Blanching'/Frozen Storage (-10°C) on Phenolics in Parsley.



The final analyte considered under the 'sealed blanching' protocol is the unknown phenolic, 'Phenolic 1', which is extensively stable to thermal treatment and subsequent frozen storage (Figure 5.15). These data confirm the likely involvement of enzymes in the degradation of this phenolic, observed in unblanched parsley. The role of 'Phenolic 1' in the oxidation systems in parsley is unclear, although it has been statistically correlated to the degradation of menthatriene and the formation of 13^2-hydroxy chlorophyll 'a'. Because of the potential 'enolic' moiety of the molecule, it might compete with the flavonoids as a substrate for peroxidase and thus form a comparable co-oxidant, or alternatively may be a target for co-oxidation.

5.3 Conclusions

Within this chapter an accelerated storage protocol (20°C) has been established and shown to reflect the volatile changes (monoterpene degradation) observed during the storage of frozen parsley (-10°C; Chapter 4.2.1), and has been applied to evaluated specific processing parameters. Extensive volatile loss, resulting from evaporation and partition into blanch water, was observed during a traditional open blanching process, however the remaining volatile compounds were stable on storage. Implementation of a novel 'sealed blanch' process has been used to establish, that the observed loss of volatiles on storage, is due to enzymic action and that oxygen is required for degradation. The oxidoreductase class of enzymes (peroxidase, lipoxygenase, polyphenol oxidase) are the most likely causative enzymes, however based on a knowledge of known substrates and oxygen requirements, it is not immediately apparent which specific enzyme might be responsible. As an alternative mode of action, the oxidoreductase have been shown to participate in coupled or co-oxidation reactions where a natural substrate of the enzyme is oxidised and in turn co-oxidises a third party species. It is concluded that co-oxidation is the most likely mode of action and used as a hypothesis for investigation in the following chapter.

The effect of thermal treatment and oxygen control have established that certain key quality chemicals (menthatriene, myrcene and chlorophyll 'a'), shown to be sensitive to frozen storage, can be stablized, with the potential to improve quality and extend shelf-life.

Chapter 6

Model Studies on Oxidoreductase Enzymes in Relation to Flavour Quality Chemicals

6.0 Introduction.

In the previous chapter, a range of endogenous species was identified as having increased stability after heat treatment, potentially resulting from the inactivation of enzymes. The requirement for oxygen in the enzymic degradation of menthatriene was also established. However, based on the functionalities of the menthatriene molecule and the known mechanisms of the oxidoreductases, no specific enzyme could be directly implicated. As a class of enzymes, the oxidoreductase have also been shown to participate in coupled or co-oxidation reactions where a natural substrate of the enzyme is oxidised, and in turn co-oxidises a third party species, for example the co-oxidation of carotenoids by lipoxygenase in bread making. Yamauchi *et al.* (1985) working with model systems proposed that apigenin, present as a major flavonoid in parsley, was oxidised by peroxidase in the presence of hydrogen peroxide to produce an 'oxidised form', capable of degrading chlorophyll although no detail of the intermediates, products or mechanism of reaction were given. Like chlorophyll, menthatriene is not a natural substrate for peroxidase due to the absence of an 'enolic' function, however, menthatriene does contain activated allylic hydrogens which are susceptible to oxidative abstraction by a suitable oxidant.

In this chapter the aim is to establish the potential role and characteristics of peroxidase and polyphenol oxidase, operating in a co-oxidative capacity, against menthatriene and chlorophyll 'a'. Within the series of model experiments performed, the objective was to validate Yamauchi's data for chlorophyll degradation and to establish the applicability of the system to menthatriene. The second objective was to identify the oxygen requirement of the menthatriene model system and determine whether the chlorophyll system followed a similar reaction pattern. The final objective was to propose a mechanism to describe the reaction sequence.

6.1 Experimental.

6.1.1 Stock Solutions.

Stock solutions were prepared using 'grade A' water and stored at 5°C (unless stated).

Saturated aqueous apigenin-7-glucoside solution (100 ml; 20°C).

80mM pH 6.5 phosphate buffer (5000 ml; 20°C).

8% w/w Triton X100 (100 ml; 20°C).

25 mM menthatriene† solution containing 0.8%w/w Triton X100 (50 ml).

75µM horse radish peroxidase solution in 80mM pH 6.5 phosphate buffer (50 ml).

9.5 mM chlorophyll 'a' in 90% aqueous acetone (1 ml).

9.5 mM lycopene in 90% aqueous acetone (1 ml).

95 mM apigenin-7-glucoside in dimethyl sulphoxide (1 ml).

95 mM umbelliferone in dimethyl sulphoxide (1 ml).

95 mM apigenin in dimethyl sulphoxide (1 ml).

95 mM naringenin in dimethyl sulphoxide (1 ml).

95 mM catechol in dimethyl sulphoxide (1 ml).

95 mM catechin in dimethyl sulphoxide (1 ml).

60 mM Hydrogen peroxide solution (100ml).

† (as parsley leaf oil, ~40% menthatriene)

6.1.2 Reagent Tables.

Table 1 Reagent Solution	Volume of Stock Solution		
	Expt 6.1.3	Expt 6.1.4	Expt 6.1.5
apigenin-7-glucoside	5 ml	1 ml	1 ml
phosphate buffer	-	3 ml	3 ml
phosphate/Triton buffer	5 ml	-	-
Triton X-100	-	20 µl	20 µl
chlorophyll (solution A)	10 ml	-	-
menthatriene	-	100 µl	100 µl
horse radish peroxidase	50 µl	10 µl	10 µl

Table 2 Reagent Solution	Volume of Stock Solution				
	Expt 6.1.6	Expt 6.1.7			Expt 6.1.8
		System 1	System 2	System 3	
apigenin-7-glucoside DMSO	10 µl	-	-	-	10 µl
umbelliferone DMSO	-	10 µl	-	-	-
apigenin DMSO	-	-	10 µl	-	-
naringenin DMSO	-	-	-	10 µl	-
phosphate/Triton buffer	4 ml	4 ml	4 ml	4 ml	4 ml
chlorophyll (solution B)	20 µl	20 µl	20 µl	20 µl	-
lycopene	-	-	-	-	20 µl
horse radish peroxidase	10 µl	10 µl	10 µl	10 µl	10 µl

Table 3 Reagent Solution	Volume of Stock Solution			
	Expt 6.1.9			
	System 1	System 2	System 3	System 4
apigenin DMSO	10 µl	-	-	-
guaiacol DMSO	-	10 µl	-	-
catechol DMSO	-	-	10 µl	-
catechin DMSO	-	-	-	10 µl
phosphate/Triton buffer	4 ml	4 ml	4 ml	4 ml
chlorophyll (solution B)	-	-	10 µl	10 µl

Reagent concentrations are nominal. Actual concentrations, based on the final reaction mixture, are given in Table 6.1 and Table 6.3.

6.1.3 Chlorophyll Degradation in a Peroxidase:H_2O_2:Apigenin-7-Glucoside Model System.

Solutions shown in the reagent table (Table 1) were pipetted into the stirred reaction vessel (20 ml / 20°C). An aliquot of the solution (1ml) was transferred to a cuvette, preloaded onto a multi cuvette carriage (sample position 1) within a UV-visible spectrophotometer (Shimadzu UV-2101PC). Distilled water (1ml) was similarly loaded into the reference cuvette (reference position) and monitored at 663nm. Hydrogen peroxide stock solution (60µl) was added to the stirred reaction mixture and data acquisition, for the previously loaded reference samples, initiated. After 20 seconds an aliquot of the reaction mix (1ml) was transferred to a third preloaded cuvette (sample position 2) and the spectrophotometer multi cuvette carriage switched from position 1 to position 2 (Figure 6.1). The complete sequence was repeated, sequentially removing one component from the model (Figure 6.1, Table 6.1)

6.1.4 Menthatriene Degradation in a Peroxidase:H_2O_2:Apigenin-7-Glucoside System.

Solutions shown in the reagent table (Table 1) were pipetted into the stirred reaction vessel (4 ml / 20°C). An aliquot of the solution (50µl) was transferred to an autosampler vial containing methanol (500µl), and chloroform (1000µl) added. The sample was analysed using the standard SNCVA protocol. Hydrogen peroxide stock solution (12µl) was added to the stirred reaction mixture and sub samples (50µl) taken with time and analysed as above (Figure 6.3, Table 6.1). The sequence was repeated, excluding peroxidase from the model (Figure 6.3).

6.1.5 Oxygen Consumption in a Peroxidase:H_2O_2:Apigenin-7-Glucoside:Menthatriene System.

Solutions shown in the reagent table (Table 1) were pipetted into the stirred oxygen electrode vessel, set to 20°C (4ml). Hydrogen peroxide stock solution (12µl) was added to the stirred reaction mixture and oxygen consumption monitored (Chapter 2, Figure 6.4, Table 6.1). The complete sequence was repeated, sequentially removing one component from the model (Figure 6.4).

Table 6.1 Peroxidase Model Systems - Reactant Concentrations

System	Data	Enzyme	Substrate 1	Substrate 2		Cooxidized Substrate		Figure
		Peroxidase	H_2O_2	Compound	[conc]	Compound	[conc]	
Yamauchi (1985)	663nm	182 nM	3.5 mM	Apigenin	16 µM	Chl 'a'[1]	13.2 µM	
Apigenin-7-G[1] Series	663nm	187 nM	176 µM	Apigenin-7-G[1]	sat[3]	Chl 'a'[1]	10 µM	6.1
Menthatriene Series	GLC	187 nM	176 µM	Apigenin-7-G[1]	sat[3]	MTriene[3]	605 µM	6.3
Menthatriene Series	O_2	187 nM	176 µM	Apigenin-7-G[1]	sat[3]	MTriene[3]	605 µM	6.4
Apigenin-7-G[1] Series	O_2	187 nM	176 µM	Apigenin-7-G[1]	236 µM	Chl 'a'[1]	43.5 µM	6.8
Umbelliferone Series	O_2	187 nM	176 µM	Umbelliferone	272 µM	Chl 'a'[1]	43.5 µM	6.10
Apigenin Series	O_2	187 nM	176 µM	Apigenin	246 µM	Chl 'a'[1]	43.5 µM	6.11
Naringenin Series	O_2	187 nM	176 µM	Naringenin	235 µM	Chl 'a'[1]	43.5 µM	6.12
Lycopene Series	O_2	187 nM	176 µM	Apigenin-7-G[1]	236 µM	Lycopene	47.5 µM	6.14

6.1.6 Oxygen Consumption in a Peroxidase:H_2O_2:Apigenin-7-Glucoside:Chlorophyll 'a' Model System .

Solutions shown in the reagent table (Table 2) were pipetted into the stirred oxygen electrode vessel, set to 20°C (4ml). Hydrogen peroxide stock solution (12µl) was added to the stirred reaction mixture and oxygen consumption monitored (Chapter 2; Figure 6.8; Table 6.1). The complete sequence was repeated, sequentially removing one component from the model (Figure 6.8).

6.1.7 Oxygen Consumption and Chlorophyll Degradation in a Peroxidase:H_2O_2:'Enolic' Compound (Various) Model System.

Solutions shown in the reagent table (Table 2) were pipetted into the stirred oxygen electrode vessel, set to 20°C (4ml). Hydrogen peroxide stock solution (12µl) was added to the stirred reaction mixture and oxygen consumption monitored (Figure 6.10-6.13; Table 6.1; Chapter 2). Chlorophyll degradation was assessed visually using a five point scale (Table 6.2). The sequence was repeated, sequentially removing one component from the model (Figure 6.10-6.13).

Table 6.2 Chlorophyll Degradation Index

Coloration after 15 minutes	Degradation Index (DI)
bright green	0
green	1
yellow green	2
green yellow	3
yellow	4
pale yellow	5
not applicable	NA

6.1.8 Oxygen Consumption in a Peroxidase:H_2O_2:Apigenin-7-Glucoside:LycopeneSystem.

Solutions shown in the reagent table (Table 2) were pipetted into the stirred oxygen electrode vessel, set to 20°C, (4ml). Hydrogen peroxide stock solution (12µl) was added to the stirred reaction mixture and oxygen consumption monitored (Chapter 2, Figure 6.14, Table 6.1). The sequence was repeated, sequentially removing one component from the model (Figure 6.14).

6.1.9 Oxygen Consumption and Chlorophyll Degradation in a Polyphenol Oxidase:Phenolic Compound (various) System.

Solutions shown in the reagent table (Table 3) were pipetted into the stirred oxygen electrode vessel, set to 20°C (4ml). Polyphenol oxidase solution (10µl) was added to the stirred reaction mixture and oxygen consumption monitored (Chapter 2, Figure 6.15, Table 6.3). Chlorophyll degradation was assessed visually using a five point scale (Table 6.2). The sequence was repeated, sequentially removing one component from the model (Figure 6.15).

Table 6.3 Poly Phenol Oxidase Model Systems - Reactant Concentrations

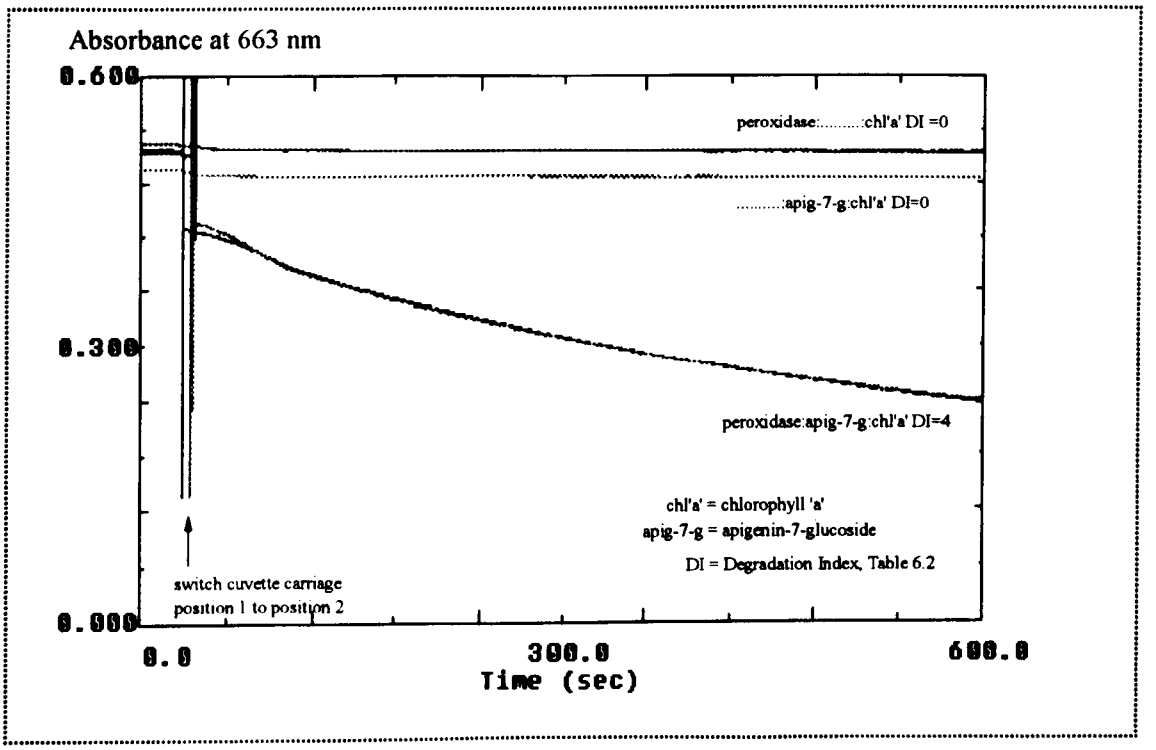
System	Data	Enzyme	Substrate 1		Cooxidized Substrate		Figure
		Poly Phenol Oxidase	Compound	[conc]	Compound	[conc]	
Catechol Series	O_2	8 nM	Catechol	235 µM	Chl 'a'[1]	43.5 µM	6.15
Catechin Series	O_2	8 nM	Catechin	235 µM	Chl 'a'[1]	43.5 µM	6.15
Apigenin Series	O_2	8 nM	Apigenin	246 µM	Chl 'a'[1]	43.5 µM	6.15
Guariacol Series	O_2	8 nM	Guariacol	175 µM	Chl 'a'[1]	43.5 µM	6.15

6.2 Results and Discussions.

6.2.1 Chlorophyll Degradation in Model Systems.

Initial experiments to validate Yamauchi *et al.* (1985) data, using the aglycone apigenin, resulted in low levels of chlorophyll degradation. However significant loss was exhibited when the glucoside, apigenin-7-glucoside was used (Figure 6.1). To establish the requirement for all elements of the model system, each individual component was sequentially replaced with a similar volume of Triton X100/phosphate buffer. From the data it can be shown that degradation only occurs if the enzyme (peroxidase), co-substrate 1 (H_2O_2) and co-substrate 2 ('enolic' flavonoid compound) are present (Figure 6.1).

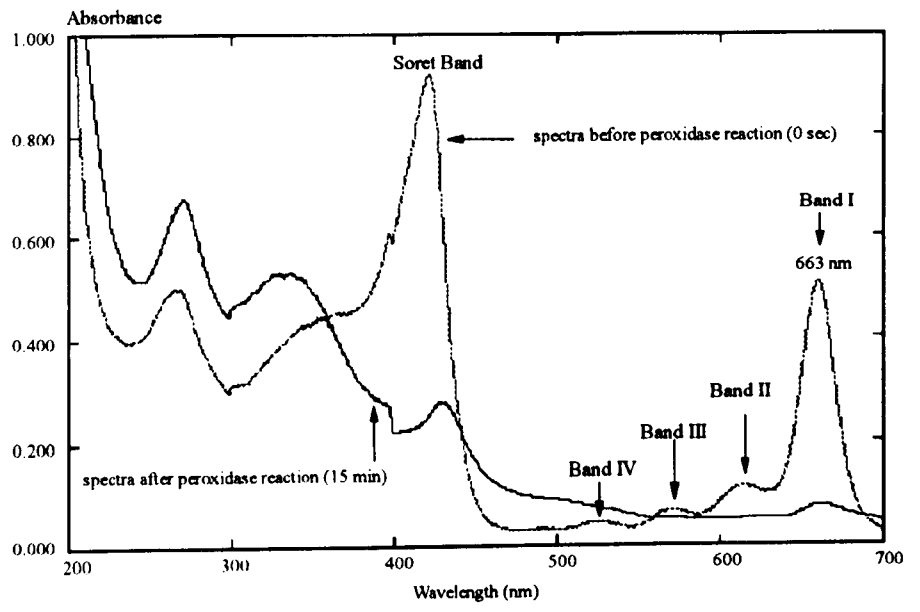
Figure 6.1 Effect of a Peroxidase:Apigenin-7-Glucoside Couple Reaction on Chlorophyll 'a' Degradation.



Visually, the reaction mixture changed colour from bright green (Degradation Index, 'DI'= 0) to yellow ('DI'= 3) after 15 minutes and continued to lose colour to yield a light straw coloration ('DI'=5) after 60 minutes (Table 6.2). The UV-visible spectra for the reaction mixture before and after the peroxidase reaction are compared in Figure 6.2. The initial chlorophyll 'a' spectrum shows characteristic absorptions at 430nm and 663nm. After reaction, the absorption at 663nm is extensively lost (~90% reduction) whilst the

absorption at 430nm is reduced to a lesser extent (70% reduction) and is shifted to a higher wavelength (442nm). Similar disproportionate loss between the Soret band and Band I have been reported in photodecomposition of chlorophyll (Jen *et al.* 1970). The green coloration of chlorophyll arises from the absorption of both blue (~ 400nm) and red (~ 650nm) light from incident white light, while the predominance of blue light absorption with little or no red absorption would produce the observed yellow coloration. If we consider the chemical functionality of the porphyrins and their associated spectral characteristics (Holt 1965; Hendry *et al.* 1987) certain information on the type of reaction can be potentially obtained. Typically, porphyrins are characterised by a Soret band at ~ 400nm and four discrete absorption bands (Band I - IV) between 500nm - 700nm (Figure 6.2).

Figure 6.2 Absorbance Specta Before and After a Peroxidase Couple Reaction.



The position and relative intensity of these bands are a function of the delocalisation of electrons via an extensive conjugated system within the molecule and are markedly affected by substituent groups which offer mesomeric effects, for example carbonyl groups, carbon double bonds, etc. Unsubstituted porphyrins typically display a low intensity absorption for Band I, however, the presence of a ketone group at C_{13}^{2} as in the

case of chlorophyll 'a', markedly increases the significance of this band. Increased delocalisation within the molecule shifts the Soret band to higher wavelengths and 'Band I' to lower wavelengths, for example the substitution of the C_7 methyl group in chlorophyll 'a' with a methanal group (negative mesomeric effect) in chlorophyll 'b', produces a wavelength shift from 430 to 465nm and 663 to 645nm respectively (Figure 1.12). The disappearance of the main absorption peaks in chlorophyll 'a' is likely to result from the disruption of the conjugated system probably via multiple oxidative attack, and may involve fission of the porphyrin ring as proposed by Hendry *et al.* (1987). Colour loss of porphyrins can also occur, by the reduction of the double bands at C_4, C_{10}, C_{14}, C_{19} again disrupting conjugation, and would result in a porphyrinogen derivative, with the maintenance of the ring structure. A potential mechanism for the degradation of chlorophyll is discussed in Section 6.2.3.

6.2.2 Menthatriene Degradation and the Role of Oxygen in Model Systems.

Application of the peroxidase:H_2O_2:apigenin-7-glucoside model to menthatriene confirmed that menthatriene acted as the co-oxidizable substrate, within the 4 component couple, in a similar fashion to chlorophyll 'a' (Figure 6.3). Evaluation of the GLC chromatograms, used to quantify menthatriene, established a similar pattern of volatile loss to that seen during measurements on parsley tissue. Although there was considerable loss of menthatriene, no new peaks were observed that corresponded to the levels of menthatriene lost. p-Cymenene, identified as a minor peak formed on frozen storage, showed some signs of increase; however due to the low reactant concentrations used in the model systems, minor peaks were difficult to quantify.

Based on the data generated earlier (Chapter 5.2.5), which showed the requirement for oxygen when menthatriene was degraded in parsley tissue, this model system was repeated with the monitoring of oxygen consumption. These data clearly established the requirement for oxygen when all components of the system were present and supported the relevance of the model system to the situation found in parsley tissue (Figure 6.4). In the absence of menthatriene from the system (and after hydrogen peroxide addition) no oxygen is consumed suggesting that oxygen does not react primarily with the apigenin-7-glucoside. However when menthatriene is subsequently added, oxygen is consumed, indicating the addition of oxygen to the menthatriene molecule (Figure 6.4).

Figure 6.3 **Effect of a Peroxidase:Apigenin-7-Glucoside Couple Reaction on Menthatriene Degradation.**

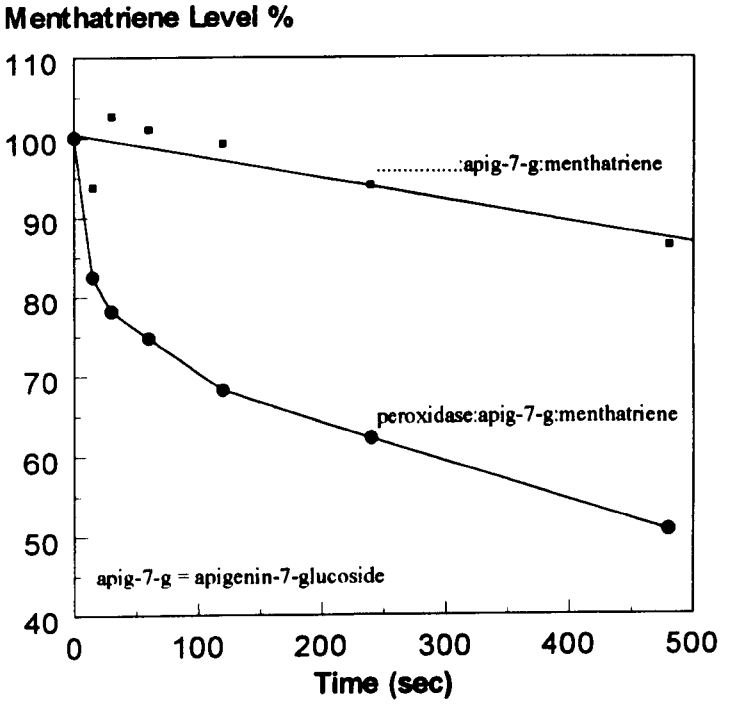
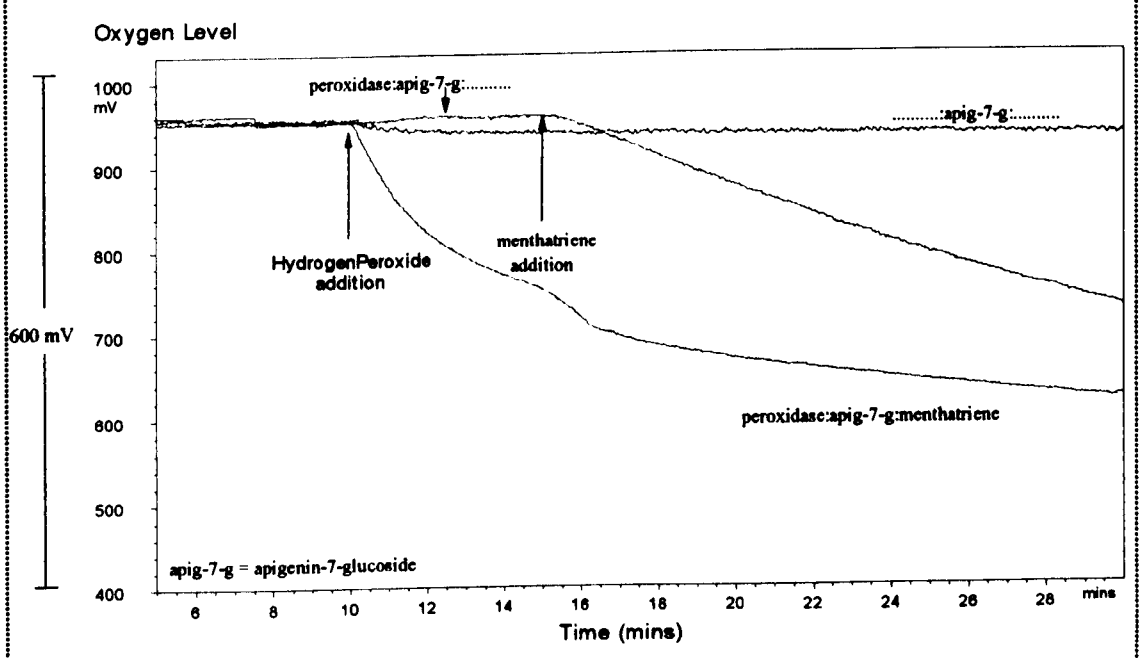
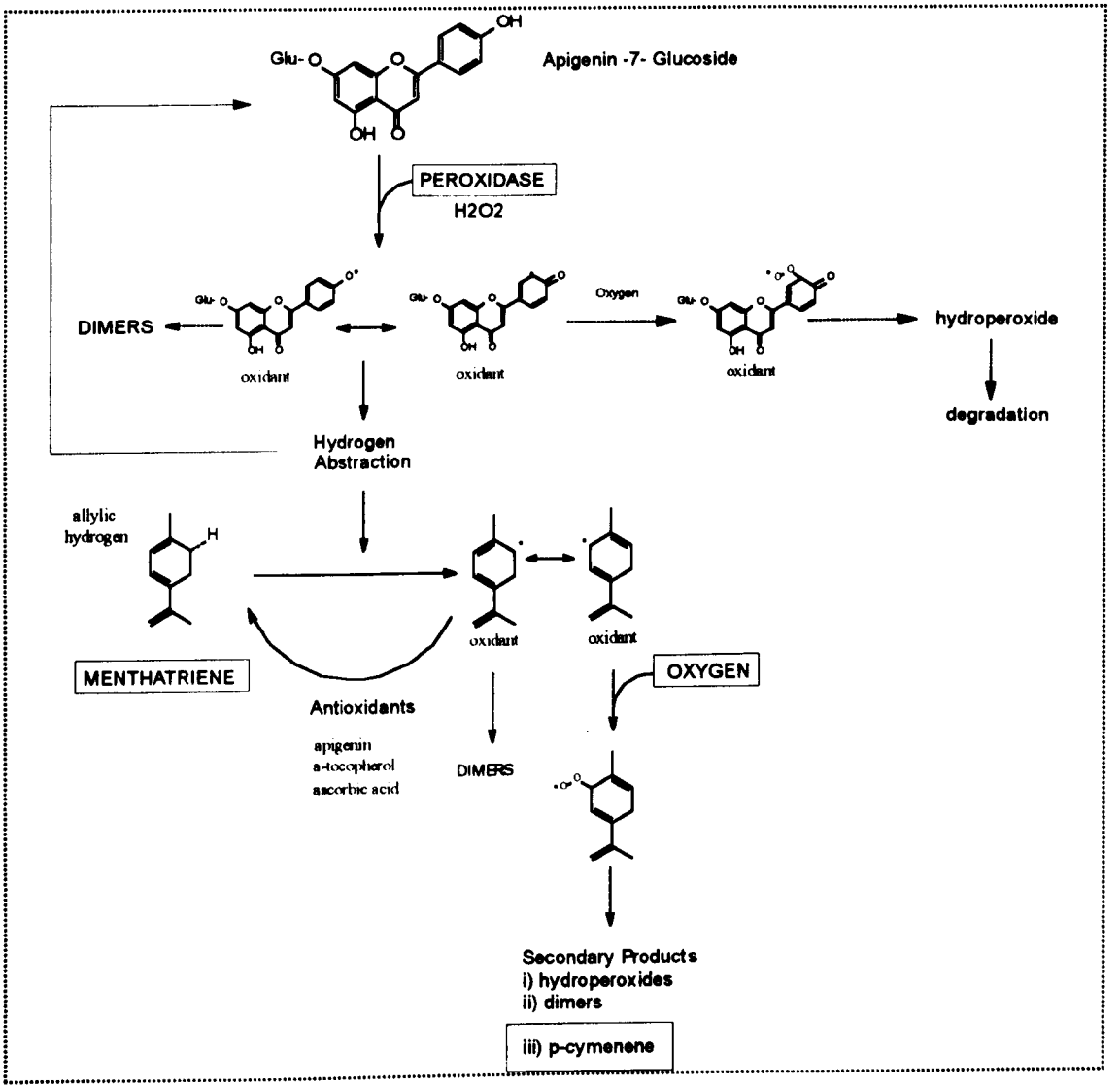


Figure 6.4 **Effect of a Peroxidase:Apigenin-7-Glucoside:Menthatriene Couple Reaction on Oxygen Consumption.**



From a knowledge of the mode of action of peroxidase, the dynamics of endogenous species in frozen parsley and the oxygen requirements established in this study, a mechanism can be proposed which identifies the order of reactions and the species involved (Figure 6.5). The proposed mechanism involves a peroxidase based oxidative abstraction of hydrogen from apigenin-7-glucoside, to generate the phenoxy radical. This radical may delocalize to produce a series of alkyl radicals at selected positions within the molecule. The radical formed is an oxidant and can take part in a series of reactions depending on the characteristics of the molecule.

Figure 6.5 Proposed Mechanism for the Peroxidase Couple Reaction with Menthatriene.



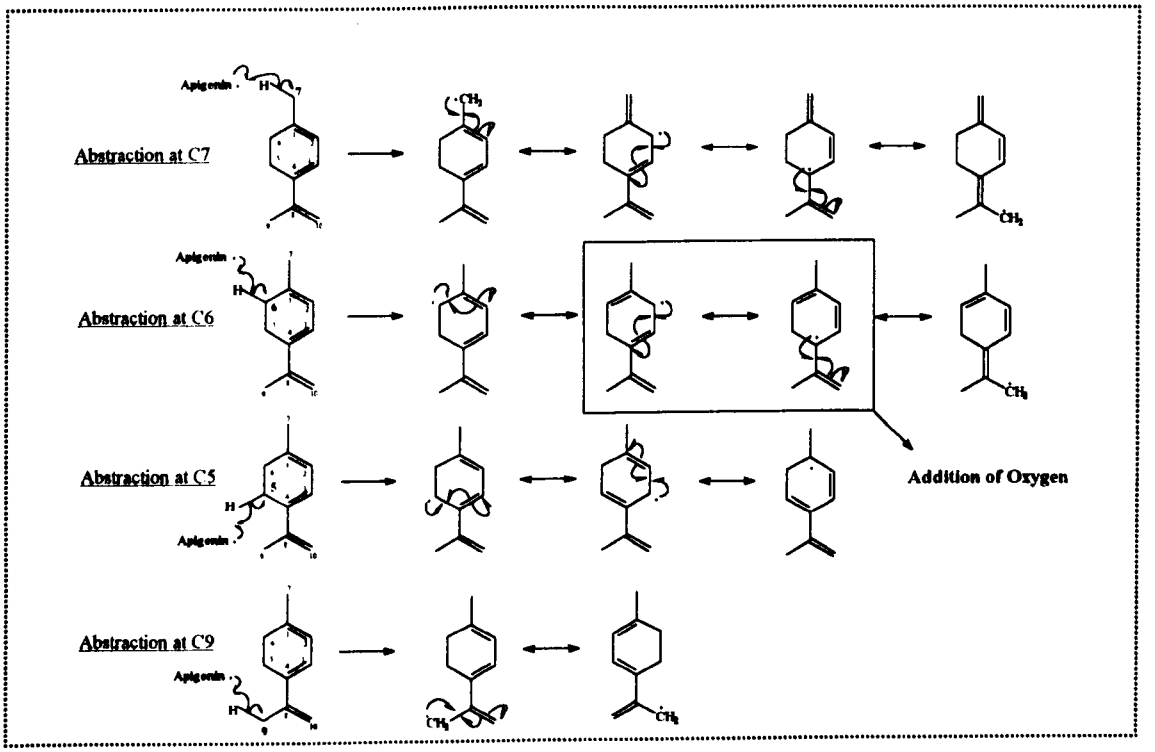
Three primary reactions are possible, dimerization and the formation of carbon-carbon or carbon-oxygen linked monomers, the addition of oxygen to the alkyl radical with the formation of the associated hydroperoxide, and thirdly, the oxidative abstraction of hydrogen from a suitable co-oxidizable molecule with the reformation of the parent apigenin-7-glucoside. In the case of menthatriene, several activated allylic hydrogens are available for abstraction which, on removal, yield the associated alkyl radical. Like the parent flavonoid radical, similar reaction options are available, however in contrast to the enzymically generated flavonoid radical, the menthatriene radical is chemically generated and not subject to enzyme specificity constraints. Moreover, reactions are governed by chemical energetics and represent the first stage of a chemical oxidative cascade initiated by a biochemical reaction.

From the information generated in this study, the preferred reactions can be established within the overall mechanism presented. In parsley tissue, the absence of oxygen allows menthatriene to be extensively stabilised. If, as the mechanism suggests, the menthatriene radical is formed, rather than dimerizing it may potentially reform by abstracting a hydrogen from a suitable antioxidant species, for example ascorbic acid. From the *in vivo* SNCNVA measurement of ascorbic acid, under frozen storage (-10°C), a rapid degradation was observed in timescales preceding menthatriene loss (Figure 4.2, 4.9). This situation is consistent with a more rapid, and preferential loss, of the antioxidant species, followed by degradation of the species the antioxidant protects and tentatively supports this rationale. However consideration must be afforded to the degradative role of ascorbic acid oxidase and other free radical systems present in the tissue, and capable of oxidizing ascorbic acid.

The formation and fate of the proposed hydroperoxides of menthatriene, generated by the addition of oxygen to the alkyl radical (Figure 6.5), are difficult to predict. However from our volatile analysis we do not observe their presence, even though the analytical protocol would be expected to be sensitive to their assessment, and hence may be considered as secondary products or reactive intermediates. The proposed reaction mechanism for the degradation of menthatriene (and the formation hydroperoxides, Figure 6.5) parallels the free radical reactions observed during chemical autoxidation, with the exception of a biochemically generated initiating radical (Chapter 1.6.1, Figure 1.10). Chemical

autoxidation reactions, involving the oxidation of fatty acids, and the formation and degradation, of hydroperoxide intermediates, have been extensively researched (Grosch *et al.* 1987; Frankel *et al.* 1982; Gardner 1975). These studies have been used as a basis to propose additional reaction schemes, relevant to menthatriene, with the aim of rationalizing the experimentation data, in relation to volatile degradation (monoterpenes) and the formation of oxidation products (p-cymenene, 1,2-3,4-diepoxide of menthatriene). In the earlier reaction mechanism (Figure 6.5), an allylic hydrogen at C6 was shown to be abstracted and the resulting radical delocalized to C2, with the corresponding shift of the adjacent carbon double bond. Oxygen was subsequently added to produce the corresponding C6 hydroperoxide. In addition to the C6 allylic hydrogen, menthatriene contains a number of other alternative allylic hydrogens (at C5, C7 and C9) which may behave similarly and would result in a range of different oxygenated products (Figure 6.6).

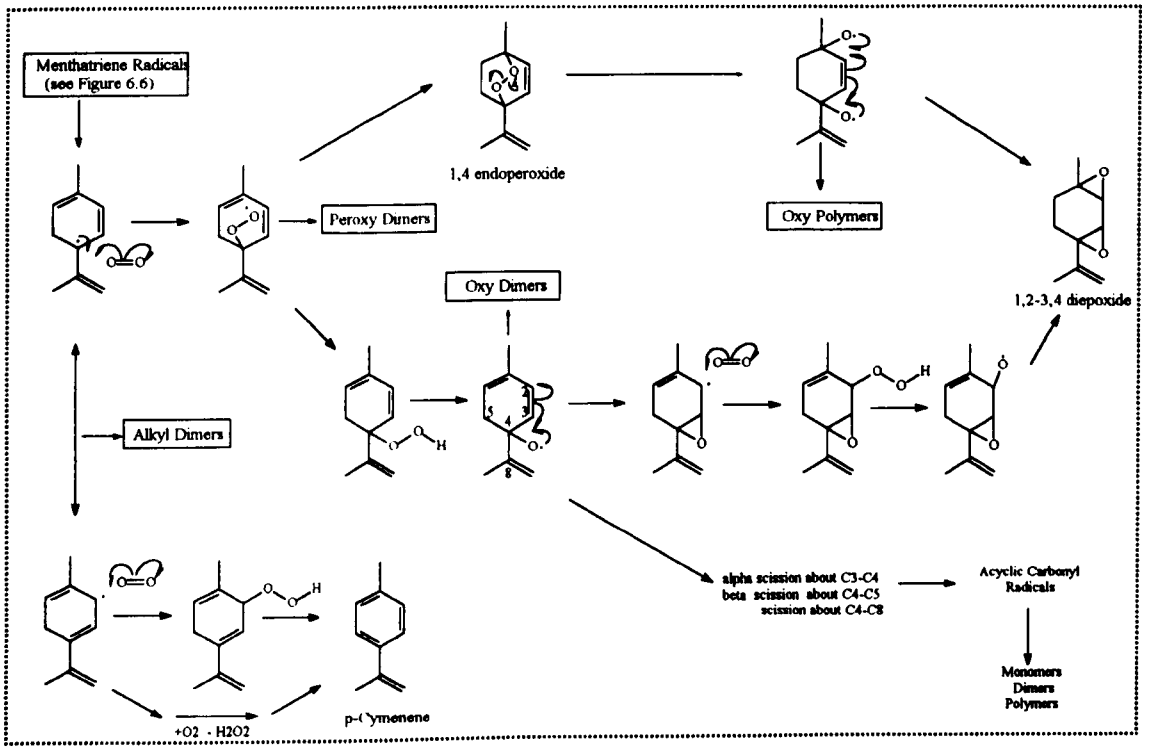
Figure 6.6 Proposed Formation of Free Radicals After the Absraction of Hydrogen from Menthatriene.



The factors that determine which hydrogen is abstracted, and the distribution of the delocalized radicals, depends on the bond energy of the carbon-hydrogen bond, the stability of the resultant radicals and the geometric energetics of the intermediate species.

These factors can be potentially estimated from theoretical molecular orbital calculations, for example *ab initio* theory with respect to electron/radical distribution, and used to predict which products or ratio of products, might be formed. These calculations would be useful to establish energetically favoured oxidation products, but also to identify which of the monoterpenes, observed to degrade (ie menthatriene > terpinolene > b-phellandrene > myrcene etc. Figure 4.2), is theoretically favoured. In this thesis, theoretical energy based molecular modelling calculations have not been performed, however radical structures are proposed, to identify possible reaction mechanisms by which monoterpenes are oxidized. These structures would form the starting point for molecular modelling studies and should be considered as part of future work. Figure 6.7 proposes a sequence of free radical reactions responsible for the degradation of menthatriene, and can be used to tentatively explain the observations made in this study.

Figure 6.7 Proposed Mechanism for the Addition of Oxygen to Menthatriene Free Radicals Formed After Hydrogen Abstraction.



Within the sequence, alkyl, peroxy and oxy dimers (and polymers) are proposed and would explain the overall loss of volatiles observed during the frozen storage of parsley. p-Cymenene can be potentially formed from the aromatization of the C2 hydroperoxide, as observed for analogous monoterpene hydroperoxides (Spraul *et al.* 1991, Matusch *et*

al. 1989,1990). The 1,2-3,4-diepoxide of menthatriene, cited in Hartmann's (1985) study and tentatively identified here (Chapter 5), may originate from either the 1,4 endoperoxide or via the sequential epoxide formation initiated by the homolytic fission of the C2/C4 hydroperoxide (Figure 6.7). Additional breakdown product may also arise from the homolytic fission of the hydroperoxide followed by alpha and beta scission of the carbon bond adjacent to the hydroperoxide attachment point. The resultant radicals could subsequently dimerize with other radicals to form high weight molecular products, which would have low volatility and as such would not be detected by SNCVA volatile analysis. It is interesting to record, that in chemical autoxidation studies involving fatty acids, much of the emphasis is placed on the generation of volatile products, however high yields of non-volatile products are observed, and would suggest dimerization and polymerization reactions are significant within such a free radical sequence (Frankel 1985). In Chapter 5.2.5 the work of Hartmann (1985) was discussed in relation to the formation of a 1,2-3,4-diepoxide of menthatriene. In the study, he suggested that photoxidation was responsible for the degradation of menthatriene, via a 1,4 endoperoxide derivative as a reactive intermediate. This reaction sequence was extensively studied in later works by members of the same research group (Technical University Munich) using model systems, however the mechanism for bioformation remained uncertain due to the endogenous requirement for singlet oxygen (Spraul *et al.* 1991; Nitz *et al.* 1989b). From the mechanism presented in this thesis, it is possible to propose a sequence by which the reported 1,4 endoperoxide and 1,2-3,4-diepoxide of menthatriene may be formed, via a free radical pathway, without the requirement for singlet oxygen. The final consideration in comparing and contrasting Hartmann's data is the disparity between the levels of 1,2-3,4-diepoxide formed relative to menthatriene loss, as discussed earlier (Chapter 4.2.1, 5.2.5). One possible explanation to account for the relatively high levels in Hartmann's work, would be the presence of peroxy dimers within the tissue, potentially formed on frozen storage, and their breakdown through homolytic fission (Figure 6.7), during their flavour analysis protocol. The analysis method utilizes a liquid-liquid extraction sequence (8 h) followed by an extract concentration step to reduce the volume from 40 ml to approximately 1 ml (42°C). Such higher MW peroxydimers would not be expected to be present within the 'volatility cut' of the SNCVA technique. This proposed formation and instability of peroxy dimers may also explain the relatively large increase in the 1,2-3,4-diepoxide formed between 75 day and 150 day storage, whilst only a relatively small

change is observed for menthatriene. Certainly these data suggest that the 1,2-3,4-diepoxide is not a direct reaction product from menthatriene.

From the earlier SNCNVA measurements of flavonoids in parsley, during frozen storage at -10°C and -20°C, the primary flavonoid peak, apigenin-7-glucoside, remains largely constant, however a second minor flavonoid peak eluting at earlier retention times with comparable spectra to the primary flavonoid, increases with frozen storage (Chapter 4, Figure 4.10). From the mechanism proposed, these data suggest that the primary flavonoid, apigenin-7-glucoside is involved in a redox cycle involving the oxidative abstraction of hydrogen by peroxidase followed by reductive hydrogen abstraction from a third party species, eg menthatriene/ascorbic acid, to reform the original flavonoid. The appearance of a second minor flavonoid with similar spectra at lower retention times would be consistent with a derived molecule having a greater polarity. Such an increased polarity might occur by the addition of oxygen, to the carbon radical formed from the delocalization of the phenoxy radical produced by the action of peroxidase, and the subsequent formation of the hydroperoxide by hydrogen abstraction (Figure 6.5). For this description to be valid, it would require a degree of consumption of oxygen (see Section 6.2.3) and a reduction in the primary flavonoid which is not immediately apparent in the results found so far. However, if we consider the formation of the polar flavonoid to be a minor product during frozen storage, it is unclear whether these changes would be observed. Although, in earlier blanching studies, a tentative relationship showing the conversion between these flavonoids was established (Chapter 5.2.6). In nature, there are many examples of redox cycles, for example the role of quinones in electron transfer and ascorbic acid in acting as a protectant species against active oxygen species (Chapter 1.6.4). In the later example, ascorbic acid serves as a reducing agent, through the availability of a hydrogen atom, and provides a termination mechanism for free radical processes. In healthy plant tissue, specific enzyme systems (dehydro ascorbate reductase, glutathione reductase) allow the reduced form of ascorbic acid to be re-oxidized, with the requirement for plant energy (Figure 1.13), and forms part of the cells balance in controlling oxygen toxicity. The proposed redox cycle involving peroxidase:hydrogen peroxide:apigenin-7-glucoside, operating under post harvest frozen storage conditions, effectively produces a free radical oxidative load which is maintained by the action of peroxidase (and the supply of hydrogen peroxide) and suitable species susceptible to

oxidative hydrogen abstraction. These species (eg. ascorbic acid, lipid, chlorophyll and other quality chemicals) are continually consumed, in the absence of energy consuming regeneration mechanisms, in an oxidative cascade sequence, potentially responsible for quality loss.

Further data on the role of flavonoids in the proposed peroxidase co-oxidation sequence is discussed in Section 6.2.3 and 6.2.4.

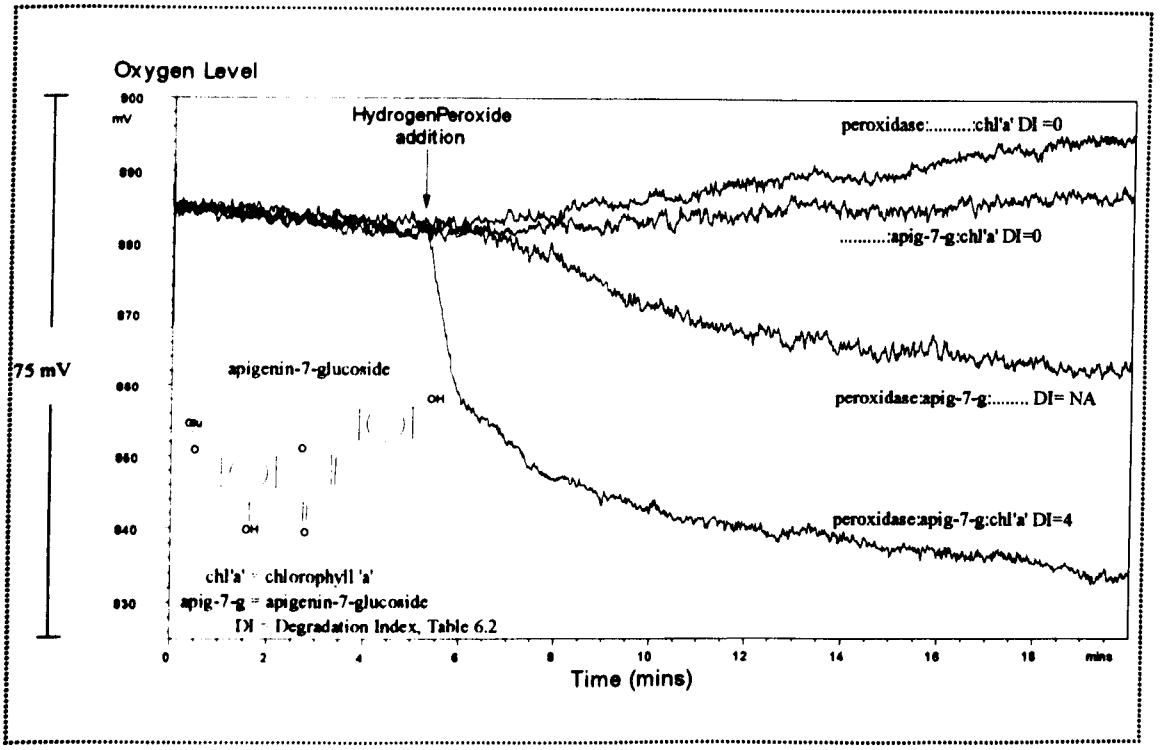
6.2.3 Role of Oxygen in the Degradation of Chlorophyll in Model Systems.

Earlier experiments on chlorophyll 'a' established the degradation in the presence of a peroxidase: apigenin-7-glucoside system, however, the role of oxygen was not established. In order to attempt to measure oxygen uptake, based on the levels consumed by menthatriene and assuming one mole of chlorophyll consumes one mole of oxygen, it was necessary to both maximise the concentration of chlorophyll 'a' in solution and maximise the rate and extent of degradation. A new protocol was established using concentrated solutions of apigenin-7- glucoside in dimethyl sulphoxide and chlorophyll in acetone/water which could be added in low volume aliquots to the aqueous reaction base containing 0.4% Triton X100/phosphate buffer (Table 6.1). This new protocol allowed the individual active elements of the co-oxidation model to be removed sequentially, for investigative purpose, and allow the levels of chlorophyll and apigenin-7-glucoside to be maximised in the final reaction mix. Previously these components were limited by the inherent dilution to form the complete reaction mixture (Reagent Table 1) and the solubility of the individual compounds in their respective solvent systems. The solubility of apigenin-7-glucoside, as with other hydroxylated flavonoids, was particularly difficult to achieve, however dimethyl sulphoxide (DMSO) was shown to be a suitable solvent. In preliminary experiments, the effect of dimethyl sulphoxide, acetone and 0.4% Triton X100 on peroxidase activity, using a guaiacol based assay, was determined and showed little or no effect on the rate of reaction.

Application of the peroxidase:H_2O_2:apigenin-7-glucoside model to chlorophyll 'a', within an oxygen electrode cell confirmed the requirement for oxygen, in a similar fashion to that observed for menthatriene (Figure 6.8). Sequential elimination of components from the

model system established a similar position to the earlier spectrophotometric data (Figure 6.1). However within this optimised protocol, low levels of oxygen consumption are observed in the absence of chlorophyll, as the co-oxidizable substrate. This situation would support a minor reaction between the flavonoid and oxygen and would account for the appearance of a polar flavonoid within the measurement *in-vivo* (ie. hydroperoxide of apigenin-7-glucoside), as discussed earlier (Section 6.2.2), and may result from the increased sensitivity of the system.

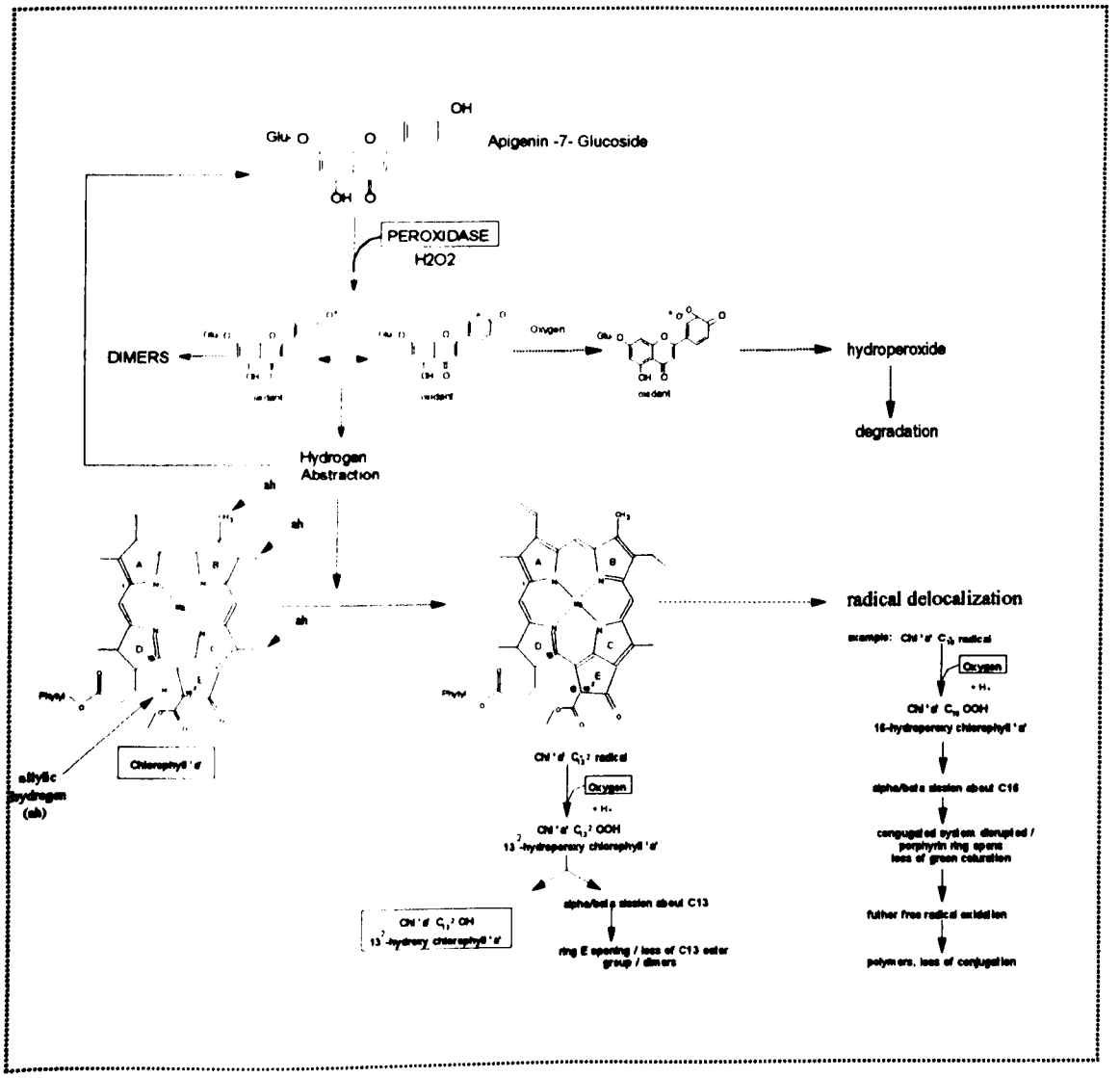
Figure 6.8 Effect of a Peroxidase:Apigenin-7-Glucoside:Chlorophyll 'a' Couple Reaction on Oxygen Consumption.



In an analogous position with menthatriene, chlorophyll 'a' also contains a number of activated allylic hydrogens, susceptible to hydrogen abstraction (Figure 6.9). In particular the allylic hydrogen at C_{13}^2 has additional bond breaking character transmitted by the mesomeric effect of the two adjacent carbonyl groups situated α,β to C_{13}^2 (Pryor 1966). Following hydrogen abstraction, to generate the C_{13}^2 alkyl radical, oxygen can add directly to form the C_{13}^2 hydroperoxide which can undergo homolytic fission and further hydrogen abstraction to generate the C_{13}^2 hydroxides, as observed (Figure 4.7). Generation of hydroxides from hydroperoxides has been reported in similar autoxidation reactions which involve unsaturated lipid (Gardner 1975). Similarly, lipid hydroxides can be formed, from

the corresponding hydroperoxide, by the enzymic action of glutathione peroxidase (Conn *et al.* 1976). Alternatively, the C_{13}^2 alkyl radical can selectively delocalise around the porphyrin ring with the possibility of oxygen addition, to produce the associated hydroperoxide. These hydroperoxides can potentially form the hydroxides, as before, or more likely degrade causing the fission of the carbon-carbon bond adjacent to the hydroperoxide attachment point, as established in autoxidation reactions (Grosch *et al.* 1987, Frankel 1985). Alternatively, the alkyl radical or the hydroperoxy radical can dimerize forming higher molecular weight species. In both situations, the porphyrin structure, determined by the conjugation system within the tetrapyrrole ring, is disrupted and as such would be expected to remove the absorption characteristic of the chlorophyll molecule (Section 6.2.1, Figure 6.9).

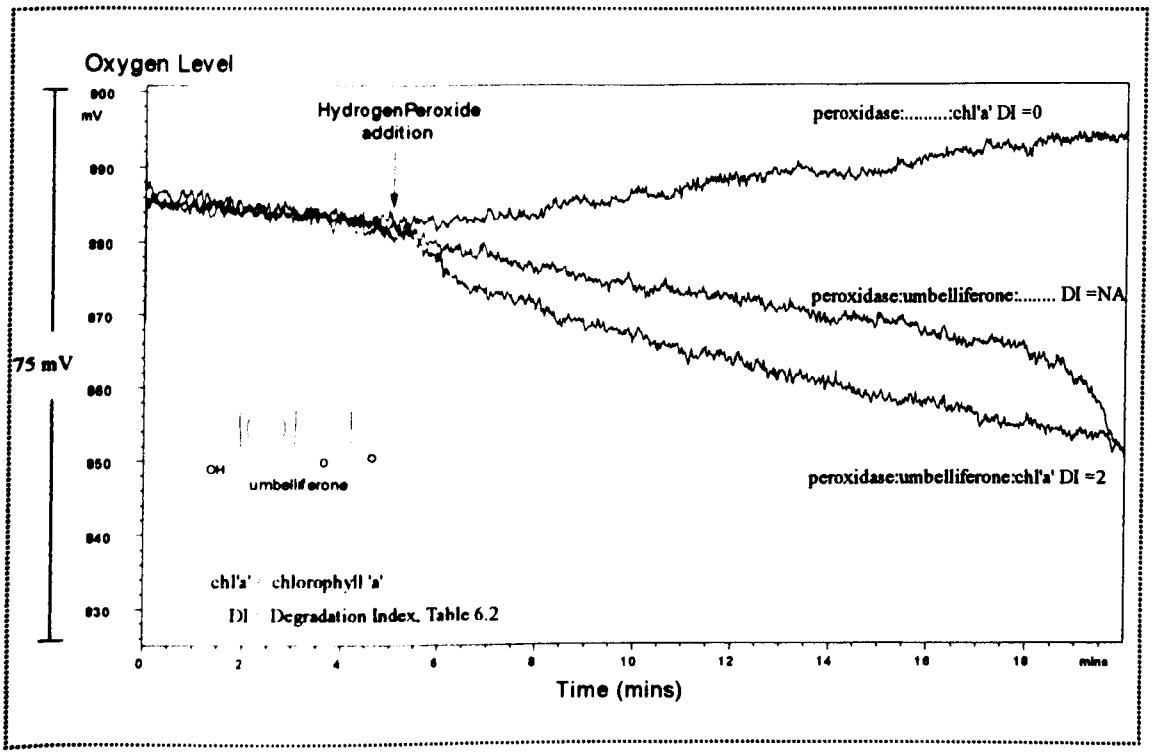
Figure 6.9 Proposed Mechanism for the Peroxidase Couple Reaction with Chlorophyll 'a'.



6.2.4 Role of Other Enolic Compounds in Co-oxidation Reactions.

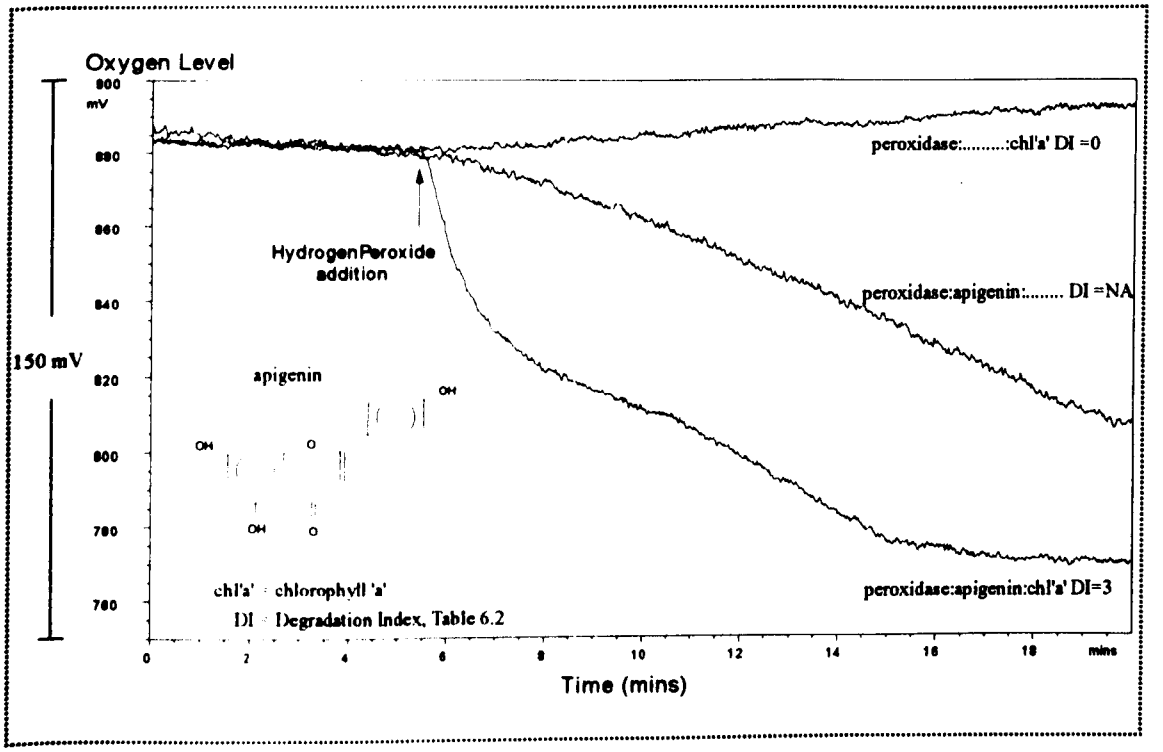
Apigenin-7-glucoside acts as a natural substrate for peroxidase and is responsible for initiating the oxidative reactions observed. In parsley, a number of other 'enolic' compounds are present which can potentially act in a similar capacity, for example the coumarin, umbelliferone. Similarly, in other tissues, such as tomato, other flavonoids analogous to apigenin are present (eg naringenin) which could be coupled to the oxidation of a range of aroma and pigment species within the tissue. Application of a peroxidase: H_2O_2: umbelliferone system to chlorophyll 'a' within an oxygen electrode cell, established the requirement for oxygen in a similar pattern displayed by apigenin-7-glucoside. Low levels of oxygen were consumed by the system in the absence of chlorophyll 'a', suggesting a minor reaction between oxygen and umbelliferone. In the presence of chlorophyll 'a' increased oxygen consumption occurs, but the degree of visual discolouration is slightly reduced compared with the apigenin-7-glucoside system (Figure 6.10). From these data it can be concluded that the coupled peroxidase reaction also applies to 'enolic' coumarins, as represented by umbelliferone, well as the flavonoids.

Figure 6.10 Effect of a Peroxidase:Umbelliferone:Chlorophyll 'a' Couple Reaction on Oxygen Consumption.



Application of a peroxidase: H_2O_2: apigenin (aglycone) system to chlorophyll 'a', utilizing the developed dimethyl sulphoxide/acetone protocol used in the previous oxygen studies, again established the requirement for oxygen during the visual discolouration of chlorophyll 'a' (Figure 6.11). Although, compared to the apigenin-7-glucoside and umbelliferone systems, a similar pattern for oxygen consumption and chlorophyll degradation was observed, considerably more oxygen was consumed by apigenin in the absence of chlorophyll 'a' and in the presence of chlorophyll 'a'. From the proposed mechanism (Figure 6.9) a significant proportion of the apigenin alkyl radical would appear to be diverted to the hydroperoxy radical with the addition of oxygen. This species would be expected to have similar oxidizing capacity to the parent radical, but would consume two molecules of oxygen compared to the single molecule for the alkyl radical route, and may account for the increased oxygen consumption observed.

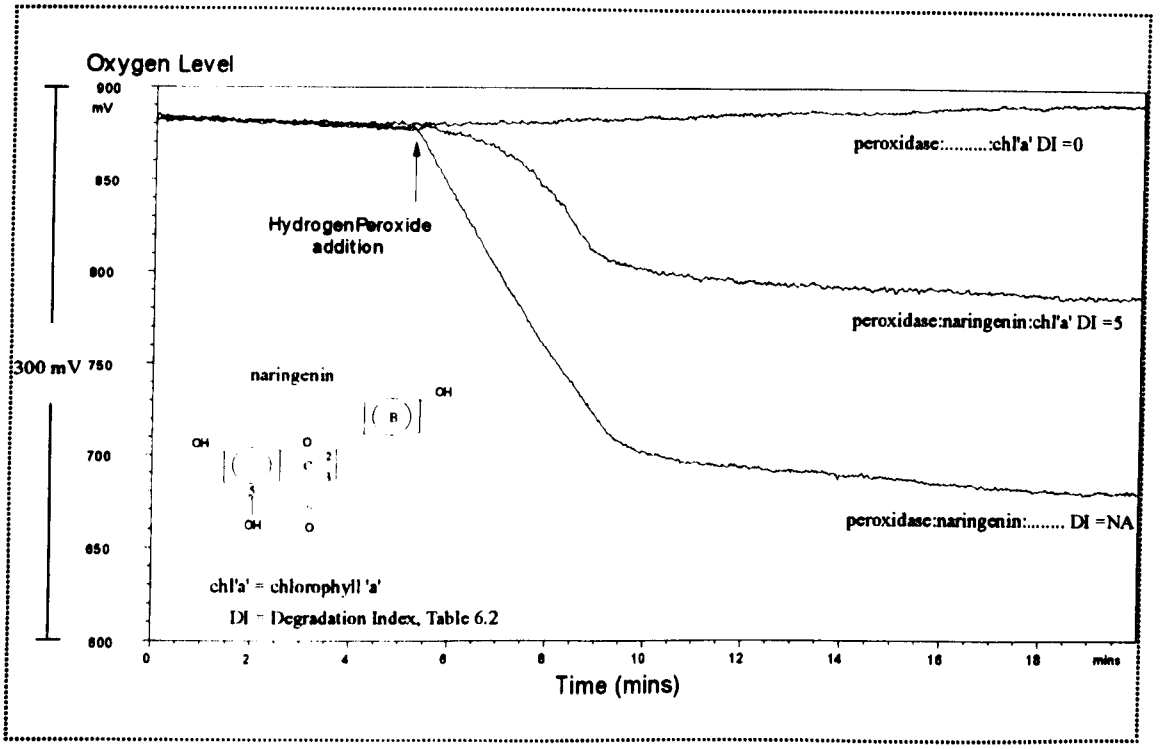
Figure 6.11 Effect of a Peroxidase:Apigenin:Chlorophyll 'a' Couple Reaction on Oxygen Consumption.



Application of a peroxidase: H_2O_2 : naringenin system to chlorophyll 'a' within an oxygen electrode cell established relatively high levels of oxygen consumption and the rapid visual discolouration of chlorophyll 'a', from bright green to yellow (Table 6.2, Figure 6.12). In contrast to the previous systems, the pattern of oxygen consumption was

significantly different. Naringenin in the absence of chlorophyll, consumed relatively high levels of oxygen, suggesting the major reaction was between naringenin and oxygen. In the presence of chlorophyll 'a', significantly less oxygen was consumed. From the mechanism proposed for apigenin-7-glucoside (Figure 6.9) it would suggest a competition for the flavonoid alkyl radial between chlorophyll and oxygen, and that the formed flavonoid hydroperoxy radical is relatively ineffective at oxidative hydrogen abstraction from chlorophyll 'a'.

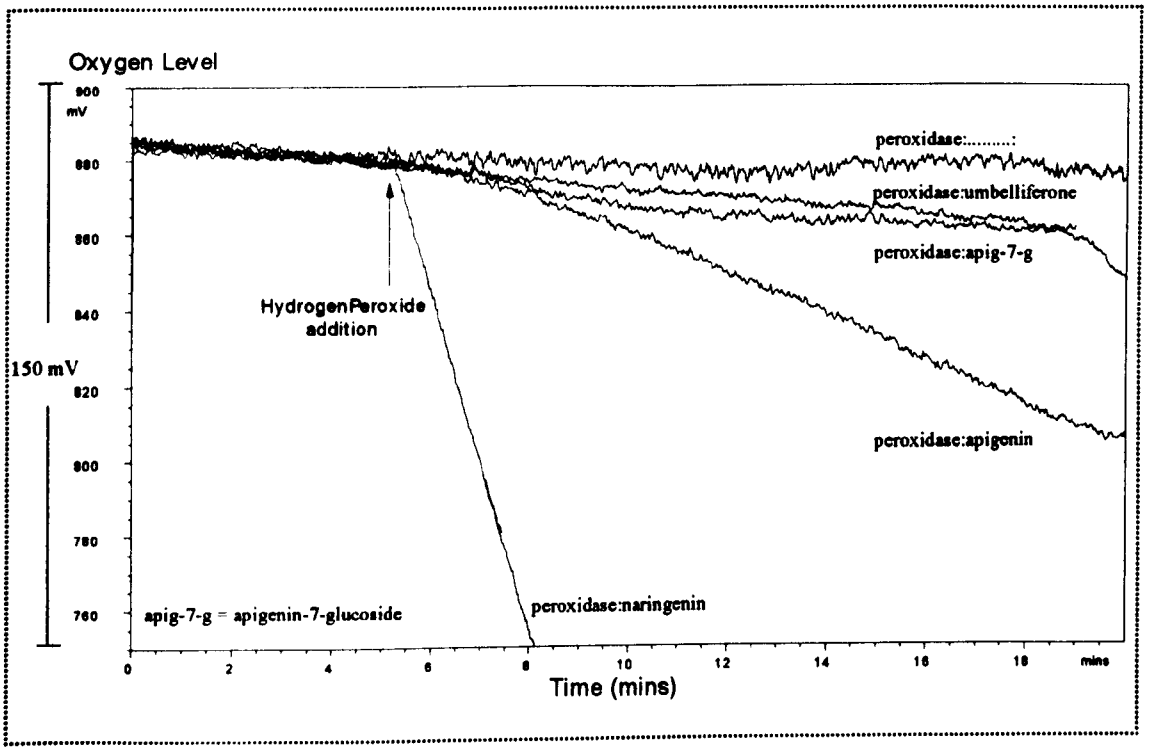
Figure 6.12 Effect of a Peroxidase:Naringenin:Chlorophyll 'a' Couple Reaction on Oxygen Consumption.



Naringenin and apigenin are structurally similar molecules, with the exception of the presence of a carbon-carbon double bond at C_2 - C_3, in the case of apigenin. Recent studies on the antioxidant properties of flavonoids have identified three primary structural components related to activity, namely the presence of an o-dihydroxy structure in ring B, a C_2 - C_3 double bond in conjugation with a 4-oxo function in ring C, and the presence of C_3 and C_5 hydroxy groups. Antioxidant character arises from the ability of a compound to firstly donate a hydrogen to the abstracting species and secondly to stabilise the resulting radical. Typically, hydrogen donation characteristics are of the same order, for an extensive range of flavonoids, however, the life span of the formed radical, as dictated

by intra molecular stabilisation, varies markedly (Bors *et al.* 1990). Contrasting the structure of naringenin with apigenin identifies, within the latter, the stabilising effect of the C_2 - C_3 double bond allowing radical delocalization through all three rings A, B and C. In view of the fact that naringenin does not possess this stabilising feature, it is in a potentially more active state and perhaps more prone to direct combination with oxygen. Additionally, the availability of oxygen, as a small molecule, to the site of radical formation would favour collision, whereas chlorophyll might be expected to be more remote and thus require a greater life-span for the oxidizing flavonoid radical. Antioxidant studies on apigenin and naringenin have been reported in separate studies. However, comparative data are difficult to assimilate (Pratt *et al.* 1990, Bors *et al.* 1990). Figure 6.13 compares the relative oxygen consumption capacity of the enolic species studied in the absence of a co-oxidizable substrate.

Figure 6.13 Comparison of a Range of 'Enolic' Substrates on Oxygen Consumption During a Standard Peroxidase Reaction.



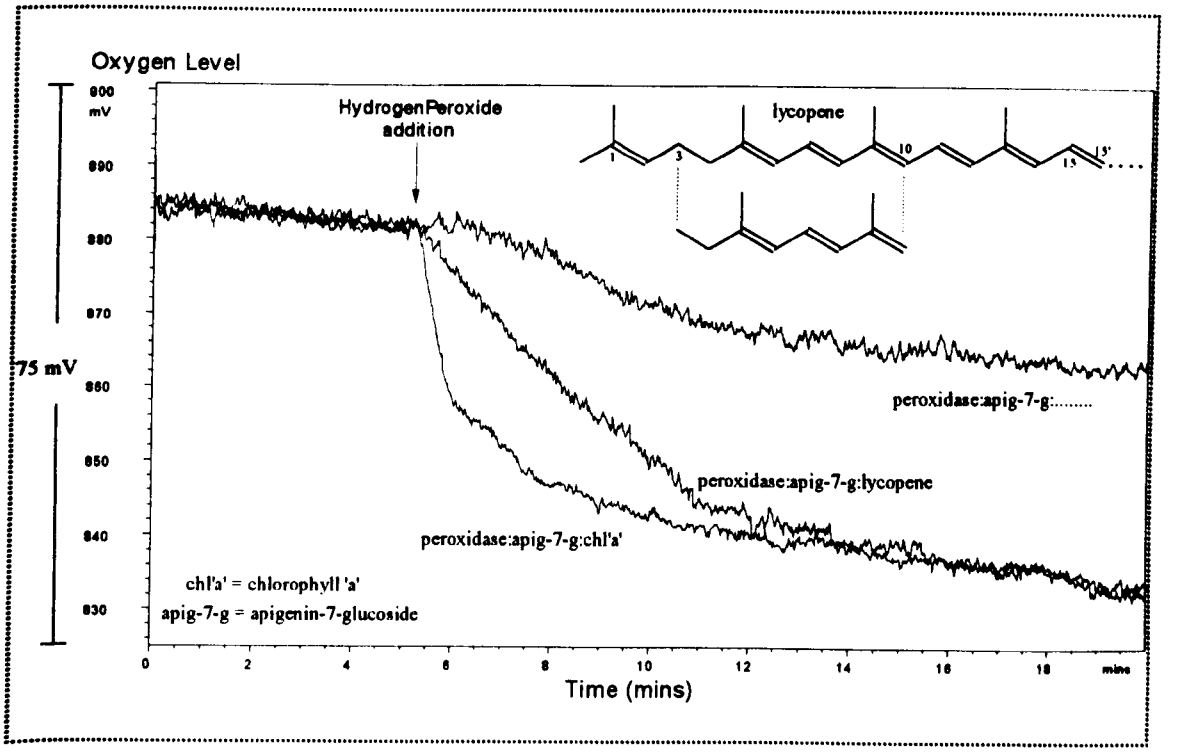
In thermally treated parsley, the polar flavonoid, identified as 'Flavonoid 2', was shown to increase significantly during the heating period (Figure 5.14). This increase may be due to the transient increase in enzymic activity, proposed as being indirectly responsible for

it's formation, and may represent an increased oxidative load within the tissue (Figure 6.5). An increase in the oxidative load may affect the dynamics of endogenous antioxidants. Ascorbic acid and violaxanthin are known to have antioxidant capacity, (Scott 1995, Sarry *et al.* 1994), and both show a very rapid decline within the initial thermal period, however the extent of thermal lability in this change is unknown (Figure 5.12, 5.13).

In parsley tissue, an unknown phenolic compound was identified, and shown to be degraded on frozen storage in unblanched tissue via an enzymic reaction (Chapter 4.2.5). Based on the reactions presented above, it would be interesting to establish if this phenolic compound is a natural substrate for peroxidase and thus a potential oxidant similar to the flavonoids, or is in fact operating as a protective species similar to ascorbic acid.

In tomato fruit, the dominant pigment is lycopene and represents a class of pigments important to both vegetable and fruits (C_{40} carotenoid). Carotenoids, like C_{10} monoterpenes are derived from isoprene sub-units, where structural similarity exists between menthratriene and the C_3 - C_{10} region of lycopene. Application of a peroxidase: H_2O_2: apigenin-7-glucoside system to lycopene, at comparable molar concentration used for chlorophyll 'a', established a similar degree of oxygen consumption, however, visual assessment of any colour change during the reaction was difficult due to the low colour intensity of the system (Table 6.1, Figure 6.14). These data suggest a reaction between lycopene and oxygen, and confirms the relevance of the peroxidative couple to the carotenoids. In Chapters 4 and 5, β-carotene and a number of xanthophylls were shown to be degraded during frozen storage of unblanched parsley, in an enzymic based reaction. The peroxidative couple is likely to apply to these compounds and may be responsible for their degradation in parsley tissue, either directly by interaction with the 'enolic' co-oxidant, as suggested in the above lycopene model, or as a protectant species similar to that proposed for ascorbic acid (Section 6.2.2).

Figure 6.14 Effect of a Peroxidase:Apigenin-7-Glucoside:Lycopene Couple Reaction on Oxygen Consumption.



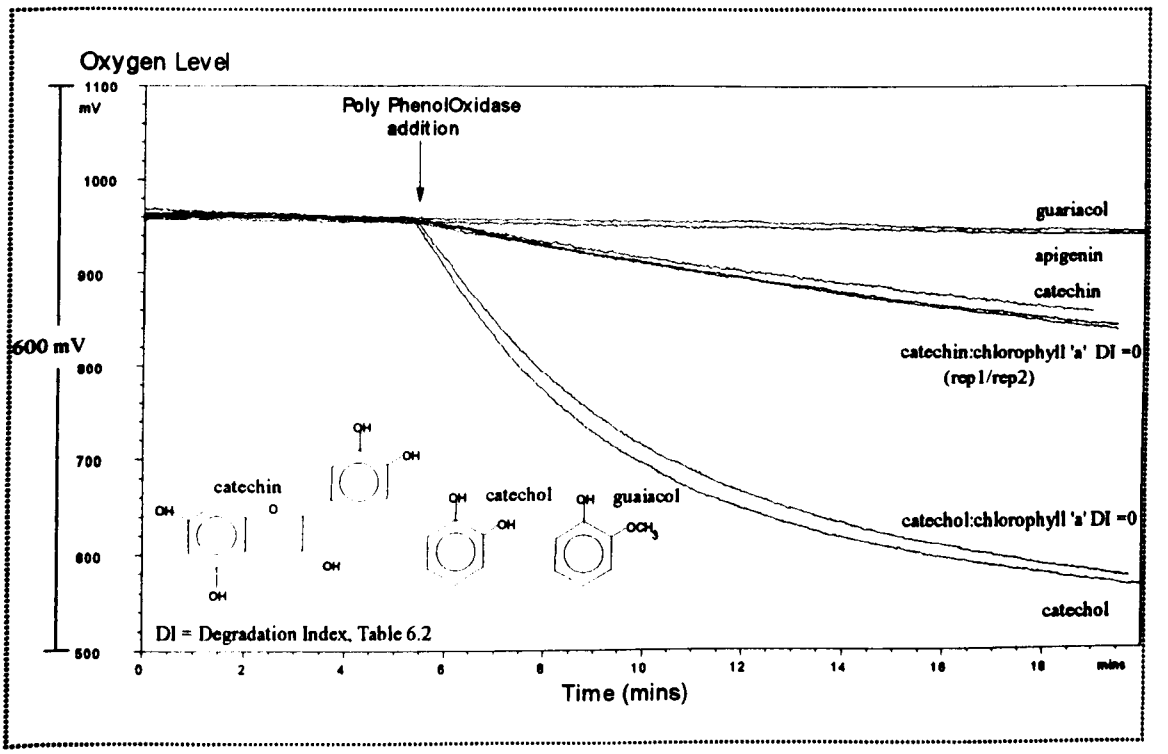
6.2.5 Role of Polyphenol Oxidase in Co-oxidation Reactions.

In parsley the co-oxidative role of peroxidase has been tentatively established. However, the relative role of the enzyme in relation to the co-oxidative roles of lipxoygenase and polyphenol oxidase has not been specifically considered (Section 1.6.5). Although lipoxygenase is known to co-oxidize carotenoids in the presence of natural enzyme substrates, in parsley there is tentative evidence to suggest that lipoxygenase activity and/or the availability of a suitable substrate are not significant, based on the low levels of C_6 volatiles generated within the tissue (Figure 4.5). This conclusion would require that the fatty acid hydroperoxide lyase enzyme within parsley was not limiting (Figure 1.9).

Polyphenol oxidase has recently been shown to bleach β-carotene and lycopene in the presence of caffeic acid. In the reaction, polyphenol oxidase is believed to convert the phenolic acid to the o-quinone which oxidizes the carotenoid (Montedoro *et al.* 1995).

Application of a polyphenol oxidase system coupled to a range of phenolic compounds, on the effect of oxygen consumption and chlorophyll degradation is shown in Figure 6.15, and Table 6.3. Catechin and catechol, in the absence of chlorophyll 'a' both consume oxygen and produce a light brown coloration of the reaction mixture as would be predicted from the polymerisation of the o-quinone. Inclusion of chlorophyll 'a' in both systems showed similar oxygen consumption levels with no visual change in green colour. From these data, chlorophyll degradation does not occur in the presence of a polyphenol oxidase/phenol couple, however, the potential role against menthratriene is unknown.

Figure 6.15 Effect of a Polyphenol Oxidase:Mono/Diphenol:Chlorophyll 'a' Couple Reaction on Oxygen Consumption.



6.3 Conclusions.

From the data presented, horseradish peroxidase has been shown to operate, *in vitro*, in a co-oxidative mode in the presence of hydrogen peroxide and a range of 'enolic' species that represent natural substrates for the peroxidase enzyme. This system can chemically co-oxidise a range endogenous chemicals including selected aroma and pigment species, via a mechanism proposed to involving the abstraction of hydrogen and addition of

oxygen. This free radical mechanism permitted the rationalisation of the data generated by SNCVA/SNCNVA, involving the formation of polymeric degradation products, loss of ascorbic acid and the appearance of minor oxidation products of menthatriene, chlorophyll and apigenin-7-glucoside.

In parsley tissue, based on the observed requirement for oxygen and the stabilizing effect of thermal treatment associated with the inactivation of peroxidase, it is believed that a similar peroxidase based co-oxidation mechanism is responsible for the oxidative degradation of monoterpenes and chlorophyll.

Oxidation of aroma and pigment species produces a change in their organoleptic and visual appearance respectively, which would be expected to affect the quality characteristics of the plant tissue from which they are delivered.

Polyphenol oxidase, proposed to operate in a similar fashion to peroxidase with mono- and di-phenols as substrates (Montedoro, *et al.* 1995), in model experiments did not effect the degradation of chlorophyll. The co-oxidative role of lipoxygenase in parsley is believed to be of minor significance, however, it is likely to be responsible for the production of low levels of hexanal observed during thawing of frozen parsley.

From these data it is concluded that the aroma and colour quality loss in frozen unblanched parsley probably results from the oxidative degradation of the unsaturated monoterpenes and chlorophyll 'a' respectively via an oxidative cascade initiated by the action of peroxidase on one or more endogenous substrates to produce a non specific oxidant. This oxidant subsequently participates in a complex series of chemically based oxidative reactions, which are responsible for the degradation of key quality chemicals and nutrients.

In view of the fact that peroxidase, 'enolic' substrates and co-oxidizable species are ubiquitous in plant tissue, it is likely that the observed degradative reactions, established in parsley, are potentially generic to vegetable and fruits.

The application of combined thermal processing and modified atmosphere offers the potential to control the degradation of key quality chemicals and in turn improve quality and extend shelf-life. From a longer term perspective strategies to control, at the genetic level, the expression of phenolic co-substrates and associated species, central to the oxidative spoilage, may offer an alternative to improve quality and extend shelf-life.

Chapter 7

Conclusions and Future Work

7.0 Conclusions

Frozen unblanched parsley was identified, from a literature review, to be a suitable tissue for investigation because of the significant colour and aroma quality deterioration displayed under typical commercial frozen storage conditions. From the review, five chemical categories were identified relevant to the quality changes observed in parsley, namely,

(i) components observed to change during technological processing of parsley.

(ii) key odour potent/quality components in parsley.

(iii) components which represent a particular chemical class/route of formation and are of significance due to their odour threshold/gravimetric standing.

(iv) secondary species associated with quality chemical change.

(v) enzymes associated with quality chemical change.

Colour quality loss was linked to chlorophyll degradation with the possible involvement of a peroxidase/flavonoid oxidative couple. Aroma quality change has been identified to relate to a loss in 'freshness' and 'grassy' character and an increase in 'haylike' off-flavour component although little information was available on the aroma chemicals responsible for change. Total aroma volatiles have been identified to decrease on frozen storage however no quantitative information on the individual species responsible were available. Menthatriene, a major parsley volatile, is cited to decrease on frozen storage with the appearance of the associated 1,4 endoperoxide. *In vitro* studies have established a mechanism involving the 1,4 addition of singlet oxygen; however it's role *in vivo* under dark storage conditions remains unclear. From the review data it was concluded that oxidative chemical and biochemical processes were most likely to be relevant to the chemical changes observed in frozen parsley.

The importance of measuring a wide range of volatile and non-volatile endogenous chemicals, in a concerted manner, to facilitate investigating the consequence and basis of

oxidation, was identified. Limitations in current analytical procedures to operating in a concerted mode were highlighted.. Within this thesis a unified strategy for the quantitative analysis of volatile and non volatile species has been developed and implemented, SNCVA/SNCNVA, permitting an extensive range of analytes to be measured from a single sample of plant leaf tissue. This unified analysis strategy aimed to provide a single stabilized solution from the plant tissue, as a means to establish the in-tissue concentrations with minimal isolation stress and freedom from artifacts. The solution extract was subsequently analyzed by GLC and HPLC as parallel separation techniques for volatile (SNCVA) and non-volatile (SNCNVA) species respectively. Characterization experiments using sweet marjoram, spinach and parsley established the techniques capacity to monitor labile species with minimal artifact formation when compared to the traditionally employed protocols of steam distillation and solvent extraction. This strategy is unique to this programme.

In-vivo analysis of a range of volatiles and non volatile analytes as a function of frozen storage was performed on parsley using this novel methodology.These data allowed the transient quality chemicals, and related species, within the tissue to be identified, along with various degradation products, and potentially forms a chemical basis to the quality deterioration phenomena in frozen parsley. From the chemical nature of the species involved the basis for chemical/biochemical change was tentatively classified into four groups:-

(i) oxidation with respect to the loss of ascorbic acid, and the formation of 13^2-hydroxy chlorophyll 'a', p-cymenene, hexanal and the tentatively assigned mentha-8-ene diepoxide.

(ii) polymerisation and the formation of high molecular weight species in relation to depletion of menthatriene, chlorophyll 'a' and carotenoids.

(iii) protonation in the formation of pheophytin 'a' from chlorophyll 'a'.

(iv) enzymic hydrolysis of chlorophyll 'a' to chlorophyllide 'a'.

To investigate the biochemical and chemical basis of the observed change for the monoterpenes, of the menthatriene type, an accelerated storage protocol was developed and combined with thermal blanching and a controlled atmosphere regime, eliminating oxygen. A 'sealed blanch' protocol was developed to prevent evaporative loss of volatiles during blanching. Thermal treatment of parsley extensively prevented the degradation of

the monoterpenes, suggesting that endogenous enzymes were responsible for the changes, as part of a biochemical sequence, and that chemical processes, in the absence of enzymes, only play a minor role. Elimination of oxygen, in the absence of blanching, prevented volatile loss, confirming the requirement for oxygen. The oxidoreductase class of enzymes (peroxidase, lipoxygenase, polyphenol oxidase) are the most likely causative enzymes, however based on a knowledge of known substrates and oxygen requirements, it was not immediately apparent which enzymes might be responsible. As an alternative mode of action, the oxidoreductase have been shown to participate in coupled or co-oxidation reactions where a natural substrate of the enzyme is oxidised and in turn co-oxidises a third party species. The hypothesis that peroxidase can operate in a co-oxidative couple with the flavonoid, apigenin-7-glucoside and hydrogen peroxide, as proposed by Yamauchi *et al.* (1985), was investigated to establish its potential role in the degradation of terpenoids and chlorophyll. In model experiments, using horseradish peroxidase, menthatriene and chlorophyll showed extensive degradation only when all components of the couple were present. In addition the requirement for oxygen was also established. From these data and a knowledge of the mode of action of peroxidase, a free radical mechanism was proposed thus permitting rationalisation of the data generated by SNCVA/SNCNVA, involving the formation of polymeric degradation products, loss of ascorbic acid and the appearance of minor oxidation products of menthatriene, chlorophyll and apigenin-7-glucoside. In similar model systems, naringenin and umbelliferone has been shown to behave in the same way, as co-substrates for peroxidase. Lycopene, with some structural similarity to menthatriene, was also susceptible to co-oxidation.

In parsley tissue, *in vivo*, based on the observed requirement for oxygen and the stabilizing effect of thermal treatment associated with the inactivation of peroxidase, it is believed that a similar peroxidase based co-oxidation mechanism is responsible for the oxidative degradation of monoterpenes and chlorophyll.

Oxidation of aroma and pigment species produces a change in their organoleptic and visual appearance respectively, which would be expected to affect the quality characteristics of the plant tissue from which they are delivered.

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The application of combined thermal processing and modified atmosphere offers the potential to control the degradation of key quality chemicals and in turn improve quality and extend shelf-life. From a longer term perspective strategies to control, at the genetic level, the expression of phenolic co-substrates and associated species, central to the oxidative spoilage, may offer an alternative to improve quality and extend shelf-life.

7.1 Future Work

The role of endogenous oxidoreductase enzymes to operate in a coupled or co-oxidative capacity in affecting the endogenous levels of key quality and nutrition chemicals is of significant importance in understanding and improving the quality and shelf-life of vegetables and herbs. Programmes which are designed to follow an oxidative cascade from biochemical initiation, through the various transient active oxygen species, to the oxidation of key quality chemicals would be of valve and the subject of future work.

Technical

- Extract and purify parsley peroxidase. Repeat model co-oxidation experiment with parsley peroxidase.
- Confirm the identity of the gas chromatographic peak at t_R=35.6, assigned within this thesis as mentha-8-ene-1,2-3,4-diepoxide.

- Investigate the proposed polymeric oxidation products from menthatriene and chlorophyll.
- Investigate the formation of lipid oxidation volatiles (eg. hexanal) and consider the effect of frozen storage.
- Investigate the role of ascorbic acid.
- Perform molecular orbital calculations to predict the hydrogen atom most susceptible to abstraction within the menthatriene molecule. To extend these calculations to predict the reactivity of the primary monoterpene hydrocarbons in parsley and relate to the observed reactivity.

Analytical

- Extend the analyte range of the SCNVA/SNCNVA protocol to include dehydroascorbate, parent lipids and polymeric products.
- Include the analysis of active oxygen species and enzyme active in concert with SNCVA/SNCNVA analysis.

Exploitation

- Optimise combined thermal treatment and modified atmosphere conditions as processing parameters to control oxidative change and improve quality.

Vegetables and Herbs

- Establish the role of peroxidase initiated co-oxidation in other plant tissues.

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Appendices

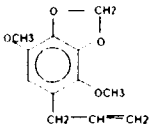
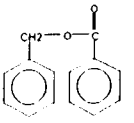
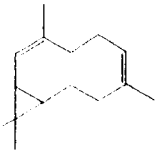
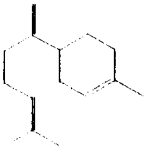
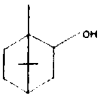
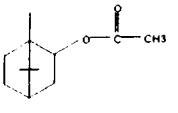
Appendix 1.1 BIOSIS indexing language applied to programme search strategy

Tissue Block		Flavour Block	
Vegetable	KW: vegetable$ (3440 hits) CC(fruits,nuts & veg)=13504 (11060 hits)	Flavour	KW: flavor$ (3030 hits)
		Taste	KW: tast$
		Aroma	KW: aroma
Tomato	KW: tomato$ lycopersicon(w)esculentum	Smell	KW: smell$
		Volatile	KW: volatil$
Potato	KW: potato$ solanum(w)tuberosum	Organoleptic	KW: organoleptic
		Terpenes	KW: terpen$ CC(sensory)=13530
Parsley	parsley petroselinum(w)crispum BC(umbelliferae)=26915	Colour	KW: color$
		Pigment	KW: pigment$
Green Bean	green(w)bean$	Texture	KW: textur$
Herbs	KW:herb$(720 hits)	Quality	KW: quality (18420 hits)
Enzyme Block		**Processing Block**	
Enzyme	CC(plant enzymes)=51518	Processing	KW:process$ (40150 hits) CC(processing)=13532
Peroxidase	EC=1.11.1.7		
Lipoxygenase	EC=1.13.11.12	Storage	KW:storag$ (10530 hits)
Polyphenol oxidase	EC=1.14.18.1	Shelf-life	KW:shelf adj life
		Frozen	KW:frozen$ KW:freez$
Catalase	EC=1.11.1.6		
Superoxide dismutase	EC=1.15.1.1	Blanching	KW:blanch$
Generation Block		**Key**	
Generation	KW:generat$ (7390 hits)	EC	enzyme commission number
Biosynthesis	KW:biosynthe$ (10680 hits)	CC	concept code
Production	KW:production	BC	biosystematic code
Phytochemistry		KW	key word

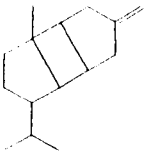
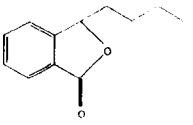
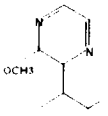
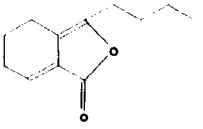
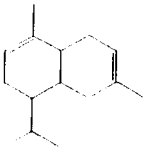
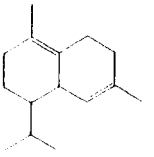
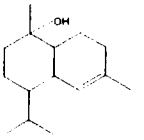
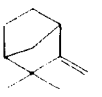
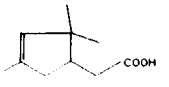
Appendix 1.2 Literature Database Summary (index: Parsley AND Volatile)

#	Author	Indexing Fields (see Table 1.1)							Rating		Title	Year	Volume	Page	Journal
130	Baardseth P	Par	Enz	Vol	Na	Na	Na	Na	***	P	Enzymes and off flavours palmitoyl coa hydrolase lipoxygenase alpha ox	1987	1 (2)	111-7	Norwegian J Agric Sci
1	Philippon J	Par	Vol	Chl	Fro	Sto	Col	Na	***	P	Storage time temperature relationships and stability of chlorophylls,	1986	6 (3)	433-46	Sci Aliments
2	Rouet-mayer	Par	Vol	Na	The	Mec	Aro	Na	***	P	Effect of blanching crumbling of leaves and packaging on the stability	1986	6 (2)	233-44	Sci Aliments
15	Freeman G;	Par	Vol	Na	Na	Na	Na	Ext	***	P	Volatile flavour components of parsley leaves	1975	26	465-70	J Sci Food Agric
22	Nitz S; Kol	Par	Vol	Na	Na	Na	Na	Pur	***	P	Oxygenated derivatives of menthatriene in parsley leaves	1989	28 (11)	3051-4	Phytochem
24	Shaath N; G	Par	Vol	Lip	Na	Na	Na	Na	***	P	Chemical composition of egyptian parsley seed absolute and herb oil	1988	715-29		Book
64	Berger R; D	Par	Vol	Na	Na	Na	Aro	Na	***	P	Natural occurrence of undecaenes in some fruits and vegetables	1985	50	1655-7	J Food Sci
116	Rouet-mayer	Par	Vol	Na	Fro	Mec	Aro	Ana	***	P	Rapid method for quantitative determination of the total volatile comp	1983	597-604		Book
128	Kim Y; Kim	Par	Vol	Na	Na	Na	Na	Na	***	P	Volatile components of parsley leaf and seed (petroselinum crispum)	1990	33 (1)	62-7	J Korean Agric Chem Soc
158	Duden R	Par	Vol	Enz	Fro	Na	Aro	Na	***	P	Enzymic lipid degradation reactions in deep frozen leafy vegetables an	1985	29 (2-4)	116-22	Agrochima
294	Jung H; Sen	Par	Vol	Na	Na	Na	Aro	Sen	***	P	Evaluation of potent odourants in parsley leaves (petroselinum crispum	1992	25	55-66	Lebensm Wiss Tech
315	Maarse H; V	Par	Vol	Na	Na	Na	Na	Na	***	P	Parsley (petroselinum crispum mill)	1989	vol 11	42 312-4	Book
406	Channell G.	Par	Vol	Chl	Fro	Sto	Qua	Ana	***	P	Influence of endogenous enzymes on the flavour quality and shelf life	1992			Comm European Comm Dgxii
420	Garnero J;	Par	Vol	Na	Na	Na	Na	Na	***	P	Contribution a l'etude de la composition chimique de l'huile essentiel	1968	11	60 332-9	La France Et Se Parfums
430	Channell G.	Par	Vol	Chl	Fro	Sto	Qua	Ana	***	P	Influence of endogenous enzymes on the flavour quality and shelf life	1993			Comm European Comm Dgxii
454	Grosch W	Par	Vol	Na	Na	Na	Aro	Sen	***		Detection of potent odourants in foods by aroma extract dilution analy	1993	4	68-73	Trends Food Sci Technolog
649	Hartmann G	Par	Vol	Na	Fro	Sto	Aro	Na	***		Aromastoffe der petersilie und deren verhalten bei verschiedenen konze	1985			Book
8	Simon J; Qu	Par	Vol	Na	Na	Na	Aro	Na	**	R	Characterization of essential oil of parsley	1988	36 (3)	467-472	J Agric Food Chem
12	Kasting R;	Par	Vol	Na	Na	Na	Aro	Ext	**	P	Volatile constituents in leaves of parsley	1972	11	2277-82	Phytochem
13	Macleod A;	Par	Vol	Na	Na	Na	Aro	Na	**	P	Volatile aroma constituents of parsley leaves	1985	24 (11)	2623-27	Phytochem
31	Porter N	Par	Vol	Na	Pre	Na	Aro	Na	**	P	Composition and yield of commercial essential oils from parsley herb o	1989	4	207-19	Flavor Frag J
62	Vernon F; R	Par	Vol	Na	Na	Na	Na	Na	**	P	Study of volatil constituents of curly parsley leaves essential oil	1983	16	32-5	Lebensm Wiss Tech
70	Fischer N;	Par	Vol	Non	Na	Na	Na	Ext	**		Applications of high speed counter current chromatography for the sepe	1991	538	193-202	J Chromatography
168	Deans S; Sv	Par	Vol	Na	The	Deh	Na	Na	**	P	Effect of microwave oven and warm air drying on the micro flora and vo	1991	3	341-7	J Ess Oil Res
216	Golubev V;	Par	Vol	Pro	Mec	Na	Na	Na	**	R	Distribution of s-methylmethionine in parsley and celeriac	1987	3	95-6	Izvestiya Vys Uch Zav Pis
225	Bernath J	Par	Vol	Non	Pre	Na	Na	Na	**	P	Production ecology of secondary plant products	1986	185-234		Book
501	Fenaroli G	Par	Vol	Enz	The	Na	Na	Na	**		Application of precursors in the flavouring of food	1975	vol 1	164-183	Book
23	Zderkiewicz	Par	Vol	Asc	Pre	Na	Na	Na	*	P	Dynamics of the accumulation of ascorbic acid and volatile oil in diff	1972	25 (2)	179-84	Acta Agrobot
29	Porter N; H	Par	Vol	Na	Na	Na	Na	Na	*		Recovery of parsley seed oil	1985	10	49-54	Perfumer Flav
32	Nitz S; Kol	Par	Vol	Na	Na	Na	Na	Na	*		Chemical changes of 1 3 8 p menthatriene in parsley	1989	12	31-32	Chem Mikrobiol Technol Le
40	Nitz S; Kol	Par	Vol	Na	Na	Na	Na	Ana	*	R	Analysis of flavours by means of combined cryogenic headspace enrichme	1988	123-35		Book
52	Lawrence B	Par	Vol	Na	Na	Na	Na	Na	*		Parsley leaf and seed oils	1989	14	54-5	Perfumer Flav
54	Lawrence B	Par	Vol	Na	Na	Na	Na	Na	*		Parsley leaf oil	1991	16	81-2	Perfumer Flav
56	Rijke D; He	Par	Vol	Na	Na	Na	Na	Na	*	R	New compounds with small rings in essential oils	1982	7	31-7	Perfumer Flav
77	Alvarez R;	Par	Vol	Na	Pac	Na	Qua	Na	*		Protection of spice quality using clear plastic packaging	1984	4 (12)	464-5	Dairy Food Sanitation
117	Johnson A;	Par	Vol	Na	Na	Na	Na	Na	*		Vegeatble volatiles a survey of components identified part 2	1971	oct	1212-24	Chem Ind (london)
119	Naik S; Len	Par	Vol	Na	Na	Na	Na	Ext	*		Extraction of perfumes and flavours from plant materials with liquid c	1989	49	115-26	Fluid Phase Equilib
126	Kraxner U;	Par	Vol	Na	Sto	Deh	Na	Na	*	P	Volatiles of parsley roots after storage under different relative humi	1980	116	47-54	Acta Hortic
151	Akhtar M; N	Par	Vol	Na	Na	Na	Na	Na	*		Physico-chemical study of the essential oil from parsley	1982	22 (2)	81-8	J Nat Sci Math
154	Naik S; Mah	Par	Vol	Na	Na	Na	Na	Ext	*	R	Separation of essential oils from plant materials by liquid carbon dio	1989	102	355-66	Vtt Symp
203	Franz C; Gl	Par	Vol	Na	Na	Na	Na	Ana	*		Tlc and glc of essential oils in leaves of some parsley varieties	1974	24 (1/2)	175-82	Qual Plant Pl Fds Hum Nut
277	Ashraf M; A	Par	Vol	Na	Na	Na	Na	Na	*	R	Studies on the essential oils of the pakistani species of the family u	1980	23 (3-4)	128-9	Pakistan J Sci Ind Res
309	Fruhwirth H	Par	Vol	Na	Pac	Sto	Na	Na	*		Essential oils in spices - percentage and method determination	1979	3	26-32	Ernahrung

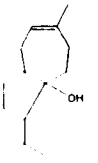
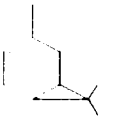
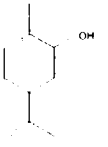
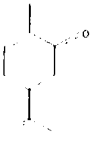
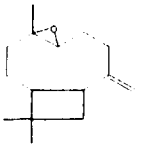
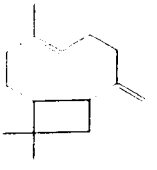
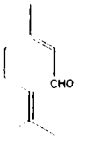
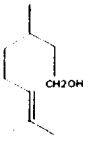
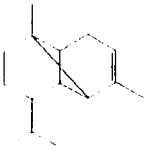
Appendix 1.3 Chemical Database Summary

Retention Index 20M / Sil5	Chemical Name / CAS No	Formula / mw / bpt	Structure	% Leaf Level / % Seed Level / Threshold	Sensory Descriptor	References (see reference list)
700 E 500	acetaldehyde aldehyde aliphatic	C2H4O 44 8		0.00	ethereal na na	12
820 500	acetone ketone aliphatic	C3H6O1 58 56		0.00	ethereal na na	12
2380 1600	apiole methoxy phenyl propanoid	C12H14O4 222 298		0.06 3.80	herbaceous warm parsley-like	128 24
  1020	benzaldehyde aldehyde aromatic	C7H6O1 106 179		0.00 6.73	sweet almond na	13
2085 1870	benzyl benzoate ester aromatic	C14H12O2 212 324		0.00	sweet balsamic floral	24 12
1721 1450	bicyclogermacrene 24703-35-3 hydrocarbon sesquiterpene	C15H24 204		0.86	unknown na na	128
1740 1512	beta-bisabolene hydrocarbon sesquiterpene	C15H24 204 262		0.46	warm spicy balsamic	128 13
1700 1167	borneol alcohol monoterpene	C10H18O1 154 204		 7.87	camphorus woody peppery	24
1600 1260	bornyl acetate ester monoterpene	C12H20O2 196 226		0.00 6.95	sweet herbaceous piney	24

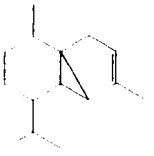
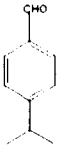
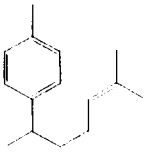
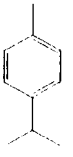
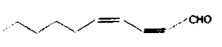
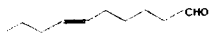
Appendix 1.3 Chemical Database Summary

Retention Index 20M / SE5	Chemical Name / CAS No	Formula / mw / bpt	Structure	% Leaf Level / % Seed Level / Threshold	Sensory Descriptor	References *see reference list*
1560 E 1255	beta-bourbonene hydrocarbon sesquiterpene	C15H24 204		0.00 0.00	unknown na na	24
2466 1639	3n-butyl phthalide ketone phthalide	C12H14O2 190 280		0.00	celery spicy herbaceous	24 820
1550 1176	2-sec-butyl-3-methoxy pyrazine methoxy pyrazine	C9H15N2O 167		0.00	musty na na	215 294
2350 1665	3-butyl-5,6-dihydro-4h-isobenzofur ketone phthalide	C12H16O2 192		0.00	spicy celery na	819 820
1780 1582	gamma-cadinene hydrocarbon sesquiterpene	C15H24 204 275		0.00	dry woody parsley-like	13
1781 1581	delta-cadinene 483-76-1 hydrocarbon sesquiterpene	C15H24 204 275		0.23	dry woody parsley-like	128
2100 1650	alpha-cadinol 481-34-5 alcohol sesquiterpene	C15H26O1 222		0.03	unknown na na	128 13
1082 938	camphene hydrocarbon monoterpene	C10H16 136 159		0.04	oily camphorus na	128 24
1400 1405	campholenic acid acid monoterpene	C10H16O2 168			unknown na na	56

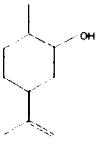
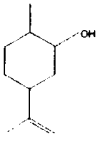
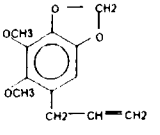
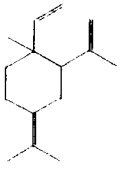
Appendix 1.3 Chemical Database Summary

Retention Index 20M / Sil5	Chemical Name / CAS No	Formula / mw / bpt	Structure	% Leaf Level / % Seed Level / Threshold	Sensory Descriptor	References *see reference list*
2100 1610	caratol 465-28-1 alcohol sesquiterpene	C15H26O1 222			unknown na na	24 12
1150 991	delta-3-carene hydrocarbon monoterpene	C10H16 136 165		0.02	sweet na na	128 24
1870 1212	l-carveol alcohol monoterpene	C10H16O1 152 227		0.65	caraway spearmint na	128
1770 1248	d-carvone ketone monoterpene	C10H14O1 150 230		6.85	warm herbaceous caraway	24
2000 1560	beta-caryophylene oxide 1139-30-6 epoxide sesquiterpene	C15H22O1 218			unknown na na	24
1610 1410	beta-caryophyllene hydrocarbon sesquiterpene	C15H24 204 256		0.41	woody spicy na	128 24
1690 1216	citral aldehyde monoterpene	C10H16O1 152 228		0.49 7.33	lemon na na	128
1750 1237	beta-citronellol alcohol monoterpene	C10H20O1 156 225		0.00 7.34	rose na na	294
1535 1359	alpha-copaene hydrocarbon sesquiterpene	C15H24 204		1.32	unknown na na	128 24

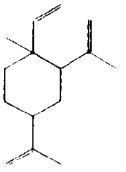
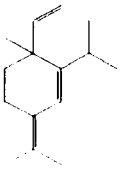
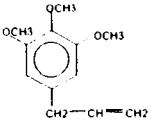
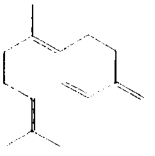
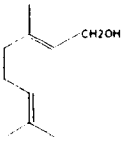
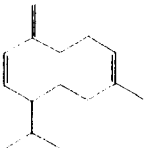
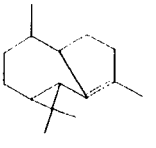
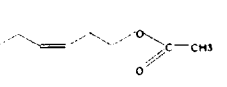
Appendix 1.3 Chemical Database Summary

Retention Index 20M / SIl5	Chemical Name / CAS No	Formula / mw / bpt	Structure	% Leaf Level / % Seed Level / Threshold	Sensory Descriptor	References *see reference list*
1485 E 1337	alpha-cubebene 31141-66-9 hydrocarbon sesquiterpene	C15H24 204		0.31	green floral estery	128 12 13
1220 900	cumene hydrocarbon aromatic	C9H12 120 152		6.92	pungent green kerosene	12 62
1770 1358	cuminaldehyde aldehyde monoterpene	C10H12O1 148 236		0.00	pungent green herbaceous	62
1780 1460	ar-curcumene 4176-17-4 hydrocarbon sesquiterpene	C15H24 204			unknown na na	24
1280 1013	para-cymene hydrocarbon monoterpene	C10H14 134 179		0.24 7.92	citrus na na	128 24
2180 1168	para-cymene-8-ol alcohol monoterpene	C10H14O1 150		0.73	apples na na	128 13
1440 1085	para-cymenene hydrocarbon monoterpene	C10H12 132		4.52	musty terpeny hay like	128 24 13 15
1835 1318	2,4-decadienal aldehyde aliphatic	C10H16O1 152		0.00 9.66	orange sweet citrus	12 294
1624 1203	E-6-decenal aldehyde aliphatic	C10H18O1 154 229			oily orange sweet	215 294

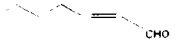
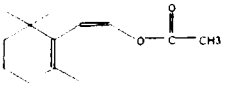
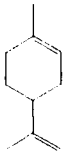
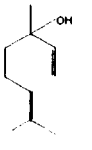
Appendix 1.3 Chemical Database Summary

Retention Index 20M / Sil5	Chemical Name / CAS No	Formula / mw / bpt	Structure	% Leaf Level / % Seed Level / Threshold	Sensory Descriptor	References *see reference list*
	diels-alder adduct (m+ 268)			0.05	na	128
		268			na	
E	unknown				na	
1890	unknown					
	diels-alder adduct (m+ 268)			0.02	na	128
		268			na	
	unknown				na	
1860	unknown					
	diels-alder adduct (m+ 268)			0.02	na	128
		268			na	
	unknown				na	
1840	unknown					
1713	trans-dihydrocarveol	C10H18O1		0.00	woody floral sweet	24
	alcohol	154				
1246	monoterpene	225				
1713	cis-dihydrocarveol	C10H18O1		0.00	woody floral sweet	24
	alcohol	154				
1244	monoterpene	225				
2380	dill apiol	C12H14O4		0.00	warm woody	24
	methoxy	222			na	
1630	phenyl propanoid	285				
1080	dimethyl disulphide	C2H6S2		0.00	cabbage	12 13
		94			na	
	sulphur				na	
638	aliphatic	112				
760	dimethyl sulphide	C2H6S1		0.00	cabbage	12 13 15
		62			na	
	sulphur				na	
520	aliphatic	37		8.23		
1620	gamma-elemene	C15H24		0.00	nutty	13 62
	29873-99-2	204			na	
	hydrocarbon				na	
1369	sesquiterpene					

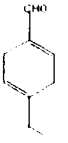
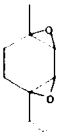
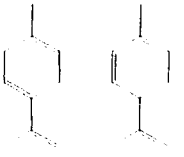
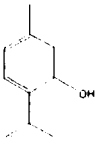
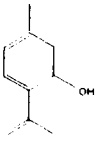
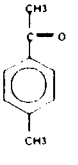
Appendix 1.3 Chemical Database Summary

Retention Index 20M / Sil5	Chemical Name / CAS No	Formula / mw / bpt	Structure	% Leaf Level / % Seed Level / Threshold	Sensory Descriptor	References *see reference list*
1620 1368	beta-elemene 33880-83-0 hydrocarbon sesquiterpene	C15H24 204		0.90 na na	nutty na na	128 62
1620 1360	alpha-elemene 5951-67-7 hydrocarbon sesquiterpene	C10H24 204		0.00	nutty na na	13
2300 1530	elemicin methoxy phenyl propanoid	C12H16O3 208 246		3.04	woody floral spicy	128 24 13
700 510	ethanol alcohol aliphatic	C2H6O1 46 78		4.26	alcoholic sweet ethereal	15
1670 1456	beta-farnesene hydrocarbon sesquiterpene	C15H24 204		0.65	sweet warm na	128 24
1830 1243	geraniol alcohol monoterpene	C10H18O1 154 230		0.21	sweet floral rose	128
1740 1480	germacrene d 23986-74-5 hydrocarbon sesquiterpene	C15H24 204		1.15	unknown na na	128 24
1400	alpha-gurjunene 489-40-7 hydrocarbon sesquiterpene	C15H24 204		0.82	unknown na na	128
1315 1010	cis-hex-3-enyl acetate ester aliphatic	C8H14O2 142 169		0.00	green fruity na	13

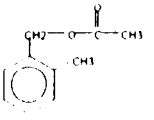
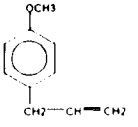
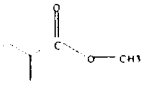
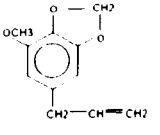
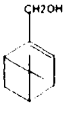
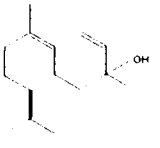
Appendix 1.3 Chemical Database Summary

Retention Index 20M / SiI5	Chemical Name / CAS No	Formula / mw / bpt	Structure	% Leaf Level / % Seed Level / Threshold	Sensory Descriptor	References *see reference list*
4000 E 1880	hexadecenoic acid acid aliphatic	C16H30O2 254 300		0.04 na na	unknown na na	128
1084 774	n-hexanal aldehyde aliphatic	C6H12O1 100 131		0.06 7.24	oily green na	128 13
1340 854	n-hexanol alcohol aliphatic	C6H14O1 102 157		0.04 6.73	winey oily fruity	128
1380 838	cis-3-hexen-1-ol alcohol aliphatic	C6H12O1 100 157		3.10	green na na	128 13 15
1220 821	trans-2-hexenal aldehyde aliphatic	C6H10O1 98		2.08 6.88	green fruity hay like	128 12
1380 846	trans-2-hexenol alcohol aliphatic	C6H12O1 100 155		0.03	fruity green caramellic	128
1955 1490	beta-ionone ketone cyclohexane	C13H20O1 192 239		0.00 na na 7.68	floral na na	294
1213 1022	limonene hydrocarbon monoterpene	C10H16 136 177		2.63 na 5.61	sweet citrus	128 24
1540 1088	linalool alcohol aliphatic	C10H18O1 154 198		0.02 6.46	floral woody citrus	128 215 294

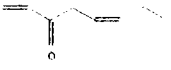
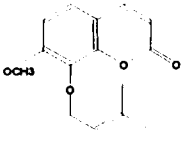
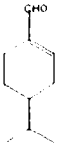
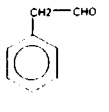
Appendix 1.3 Chemical Database Summary

Retention Index 20M / Sil5	Chemical Name / CAS No	Formula / mw / bpt	Structure	% Leaf Level / % Seed Level / Threshold	Sensory Descriptor	References *see reference list*
1820 E 1358	para-mentha-1,4-diene-7-al aldehyde monoterpene	C10H14O1 150 237		0.00	spicy herbaceous dill	62
2300 1097	para-mentha-8-ene-1,2-3,4-diepoxide epoxide monoterpene	C10H14O2 166		0.00	unknown na na	22
2300 1096	para-mentha2,8diene1,4endoperoxide endoperoxide monoterpene	C10H14O2 166		0.00	unknown na na	22
1370 1098	para-menthatriene hydrocarbon monoterpene	C10H14 134		0.06	unknown na na	128 24
1370 1115	ara-1,3,8-menthatriene hydrocarbon monoterpene	C10H14 134		10.4	spearmint terpeny na	128 24 294
2300 1185	para-menthatrienol alcohol monoterpene	C10H14O1 150		3.05	unknown na na	128 24
2300 1110	para-menthatrienol alcohol monoterpene	C10H14O1 150		0.14	unknown na na	128 24
890 500	methanol alcohol aliphatic	C1H4O1 32 65		0.00 3.73	alcoholic na na	12 13
1750 1160	para-methyl acetophenone ketone aromatic	C9H10O1 134 228		0.61 7.43	pungent sweet floral	128 13

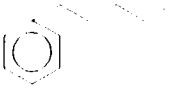
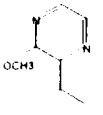
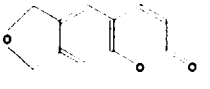
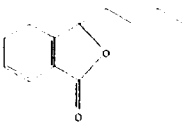
Appendix 1.3 Chemical Database Summary

Retention Index 20M / Sil5	Chemical Name / CAS No	Formula / mw / bpt	Structure	% Leaf Level / % Seed Level / Threshold	Sensory Descriptor	References *see reference list*
1810 1700	o-methyl benzyl acetate ester aromatic	C10H12O2 164 220		0.06	fruity sweet na	128
1700 1181	methyl chavicol methoxy phenyl propanoid	C10H12O1 148 216			sweet herbaceous anise	128
1000 780	methyl-2-methyl butanoate ester aliphatic	C6H12O2 116		0.00	pungent ethereal fruity	215 294
1165 984	myrcene hydrocarbon monoterpene	C10H16 136 167		3.01	sweet balsamic resinous	128 24 294 13
2245 1496	myristicin methoxy phenyl propanoid	C11H12O3 192 250		21.8	warm balsamic woody	128 24 294
1683 1180	myrtenol alcohol monoterpene	C10H16O1 152 224			warm herbaceous medicinal	62
2000 1527	alpha-nerolidol alcohol sesquiterpene	C15H26O1 222 276		0.03	woody floral green	128 24
916 855	nonane hydrocarbon aliphatic	C9H20 128 150		0.00	unknown na na	12
1275 1024	trans-beta-ocimene hydrocarbon monoterpene	C10H16 136 177		0.00	warm herbaceous floral	13

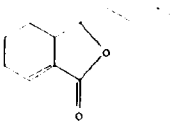
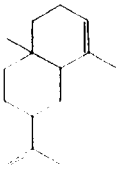
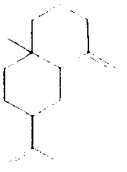
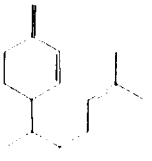
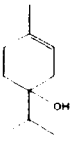
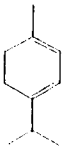
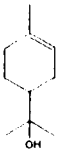
Appendix 1.3 Chemical Database Summary

Retention Index 20M / Sil5	Chemical Name / CAS No	Formula / mw / bpt	Structure	% Leaf Level / % Seed Level / Threshold	Sensory Descriptor	References *see reference list*
1242 E 1023	cis-beta-ocimene hydrocarbon monoterpene	C10H16 136 177		0.03	warm herbaceous floral	128 24
1600 985	(Z)-1,5-octadien-3-one ketone aliphatic	C8H12O1 124		0.00	floral na na	294
1315 981	1-octene-3-one ketone aliphatic	C8H14O1 126		0.00	mushrooms na na	294
2800 2000	osthol methoxy coumarin	C15H16O3 244		0.00	hay like na na	277
1211 1000	pentyl furan ether heterocyclic	C9H14O1 138		7.04	fruity na na	13
1760 1080	phellandral aldehyde monoterpene	C10H16O1 152 174			green herbaceous na	62
1230 1019	beta-phellandrene hydrocarbon monoterpene	C10H16 136 178		9.96	peppery minty citrus	128 24
1180 995	alpha-phellandrene hydrocarbon monoterpene	C10H16 136 175		0.72	citrus peppery minty	128 12
1670 1090	phenyl acetaldehyde aldehyde aromatic	C8H8O1 120 206		0.00	green floral sweet	13

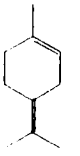
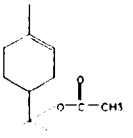
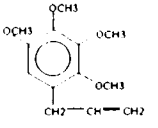
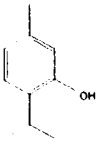
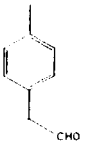
Appendix 1.3 Chemical Database Summary

Retention Index 20M / Sil5	Chemical Name / CAS No	Formula / mw / bpt	Structure	% Leaf Level / % Seed Level / Threshold	Sensory Descriptor	References *see reference list*
1380 E 1099	phenyl pentane hydrocarbon aromatic	C11H16 148		0.00 na na	unknown na na	24
1125 969	beta-pinene hydrocarbon monoterpene	C10H16 136 166		0.40	dry woody piney	128 24
1035 932	alpha-pinene hydrocarbon monoterpene	C10H16 136 157		0.83 5.41	warm resinous piney	128 24
1665 1095	4-iso-propyl cyclohex-2-en-1-one ketone cyclohexane	C9H14O1 138 198		0.07	woody minty sweet	128 24
1400 1098	2-iso-propyl-3-methoxypyrazine methoxy pyrazine	C8H13N2O 152		0.00	musty na na	294
2500 1740	psoralen ketone coumarin	C11H6O3 186		0.00	coconut sweet na	818
1130 966	sabinene hydrocarbon monoterpene	C10H16 136 165		0.09	warm oily woody	128 24
1082 930	santene hydrocarbon monoterpene	C9H14 122 40		0.00	camphorus balsamic na	277
2543 1707	sedanenolide ketone phthalide	C12H16O2 192		0.00	celery spicy na	24 820

Appendix 1.3 Chemical Database Summary

Retention Index 20M / Sil5	Chemical Name / CAS No	Formula / mw / bpt	Structure	% Leaf Level / % Seed Level / Threshold	Sensory / Descriptor	References *see reference list*
2514 E 1723	sedanolide ketone phthalide	C12H18O2 194 284		0.00	spicy herbaceous celery	24 820
1770 1484	alpha-selinene hydrocarbon sesquiterpene	C15H24 204 258			sweet woody peppery	24
1770 1483	beta-selinene hydrocarbon sesquiterpene	C15H24 204 269		0.28	woody warm herbaceous	128 24
1780 1520	sesquiphellandrene 20307-83-9 hydrocarbon sesquiterpene	C15H24 204		0.00	unknown na na	24 62
1615 1173	terpinen-4-ol alcohol monoterpene	C10H18O1 154 212		0.12 5.63	peppery woody green	128 24
1260 1077	gamma-terpinene hydrocarbon monoterpene	C10H16 136 183		0.07	herbaceous citrus na	128 24
1210 1009	alpha-terpinene hydrocarbon monoterpene	C10H16 136 176		0.04	lemon citrus na	128 24
1700 1179	alpha-terpineol alcohol monoterpene	C10H18O1 154 219		0.45 6.62	floral sweet na	128 24
1320 1083	alpha-terpinoline hydrocarbon monoterpene	C10H16 136 184		5.51	sweet piney oily	128 24

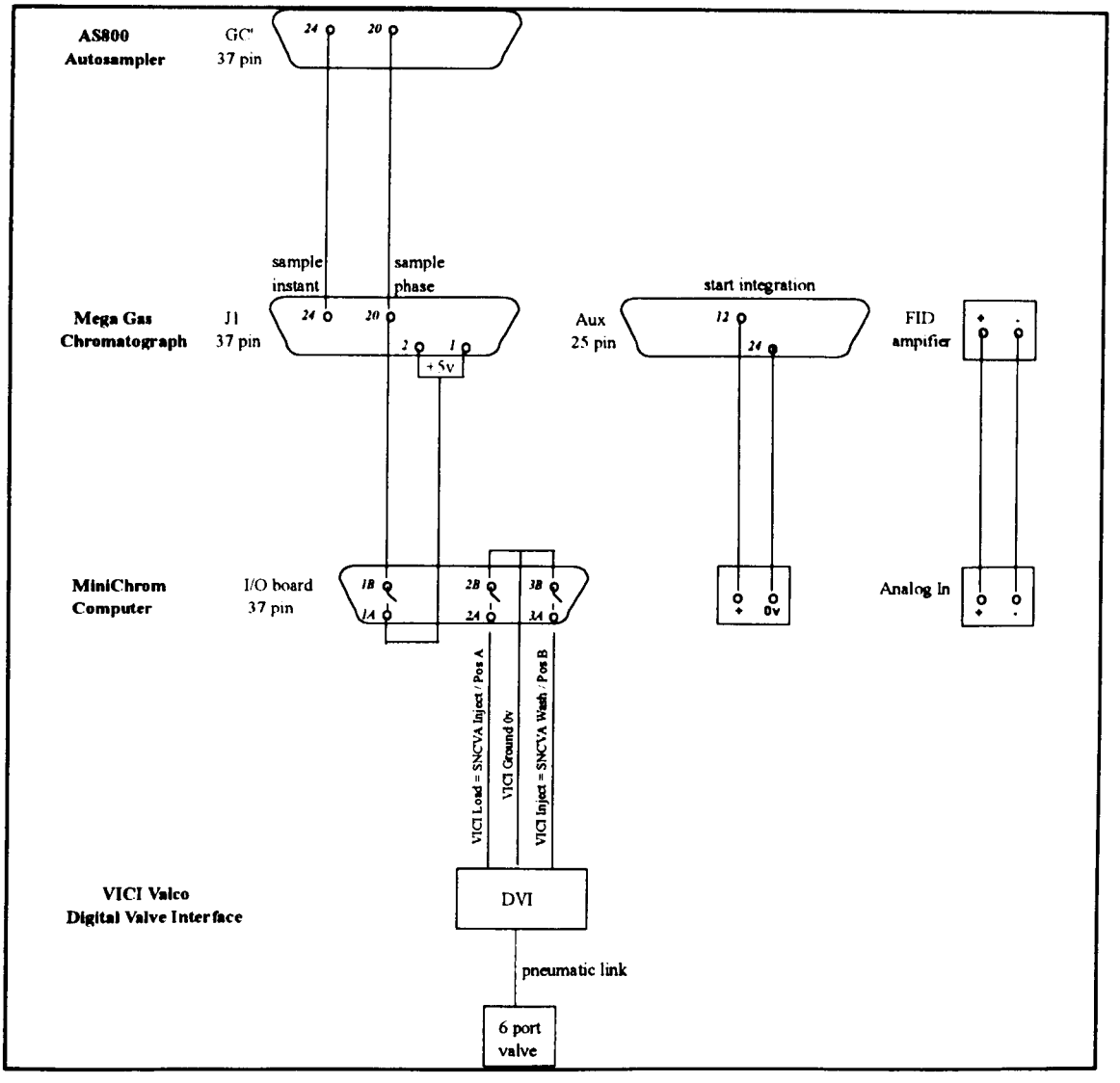
Appendix 1.3 Chemical Database Summary

Retention Index 20M / Sil5	Chemical Name / CAS No	Formula / mw / bpt	Structure	% Leaf Level / % Seed Level / Threshold	Sensory Descriptor	References *see reference list*
1320 E 1083	alpha-terpinoline hydrocarbon monoterpene	C10H16 136 184		5.51	sweet piney oily	128 24
1722 1250	alpha-terpinyl acetate ester monoterpene	C12H20O2 196 220		0.00	herbaceous sweet spicy	24
1900 1820	tetradecanal aldehyde aliphatic	C14H28O1 212 260		0.64	fruity citrus amber	128
2380 1590	1,2,3,4-tetramethyl alkyl benzene hydrocarbon phenyl propanoid	C13H18O4 159			unknown na na	24 13
1035 928	alpha-thujene hydrocarbon monoterpene	C10H16 136		0.02	unknown na na	128 24
2300 1094	thymol alcohol monoterpene	C10H14O1 150 233		0.00 7.02	sweet medicinal herbaceous	8
1770 1174	2(para-tolyl) propanal aldehyde monoterpene	C10H12O1 148			unknown na na	13 62
1140 1092	1-EZ,3,5-undecatriene hydrocarbon aliphatic	C11H18 150		0.00	fruity balsamic na	64
1230 987	para-xylene hydrocarbon aromatic	C8H10 106		0.00 5.67	green kerosene na	13 15

References for Appendix 1.3

Database Ref No	Reference
8	Simon *et al.* (1988).
12	Kasting *et al.* (1972).
13	MacLeod *et al.* (1985).
15	Freeman *et al.* (1975).
22	Nitz *et al.* (1989a).
24	Shaath *et al.* (1988).
56	Rijke *et al.* (1982).
62	Vernon *et al.* (1983).
64	Berger *et al.* (1985).
128	Kim *et al.* (1990).
215	Grosch (1990).
294	Jung *et al.* (1992).
277	Ashraf *et al.* (1980).
819	Nitz *et al.* (1992).
820	Guth *et al.* (1993).

Appendix 3.1 SNCVA II Control System - Hardware



Appendix 3.2 SNCVA II Control System - Software

Control Sequence : Fisons MiniChrom Digital EventsControl Table.

Comment	Line	Time	Switch	Repeats	Rep Time
Valve to Wash Position	2	19.00	CLOSE	0	0.00
	2	19.10	OPEN	0	0.00
Wash Injection (x3)	0	66.00	CLOSE	2	5.00
	0	66.10	OPEN	2	5.00
Valve to Inject Position	1	104.80	CLOSE	0	0.00
	1	104.90	OPEN	0	0.00

AS800 Analysis Methods (SNCVA)

Function	Task	Sample Method A1	Rinse Method A2
Analysis Time		3 min	2 min
Syringe Pre Clean Conditions	Continue (repeats)	3	1
	Mode	INJ	SMP
	Volume(µl)	8.0	10.0
	Wash Solvent (S1)	ä	ä
Sample Conditions	Sample Volume (µl)	2.0	9.8
	Air Volume (µl)	0.2	0.2
	Fill Volume (µl)	8.0	10.0
Fill Conditions	Clean (repeats)	3	1
	Bubble Exclusion (repeats)	3	2
	Fill Rate (µls)	1	1
	Delay after fill D1 (s)	3	1
Injection Conditions	Plunger Rate (µls)	5	5
	Pre Injection delay (s)	15	30
	Post Injection Delay (s)	15	15
Syringe Post Clean Condition	Continue (repeats)	5	1
	Mode	INJ	SMP
	Volume(µl)	8.0	10.0
	Wash Solvent (S1)	ä	ä

Fisons Instruments - AS800 manual Part No 317.09084